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ALZHEIMER'S DISEASE: AMYLOID OLIGOMERS, THERAPEUTIC AGENTS, THEIR TARGETS, AND MODE OF ACTION

INAUGURAL-DISSERTATION
ZUR ERLANGUNG DES DOKTORGRADES DER HUMANBIOLOGIE
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ஈன்றபோது பெரிதுவந்த எனதன்பு
தாய்தந்தையர்க்கு எப்போதும் இனிதுவக்க யான்
செய்யும் சிறு சமர்ப்பணம்..

(in Tamil)

Dedicated to my parents..

1 INTRODUCTION

1.1 Alzheimer's disease

In the year 1907, two scientists (Alois Alzheimer and Oskar Fischer) (Figure 1) simultaneously described the presence of plaques and tangles in the brains of elderly people. Alzheimer published a short report about senile plaques observed in his patient Auguste Deter's post-mortem brain (Alzheimer, 1907). Fischer published a clinico-pathological study of 16 cases of senile dementia describing the neuritic plaques (Fischer, 1907). Later, in the year 1910, the disease was named after the Bavarian psychiatrist as 'Alzheimer's disease' (AD) by his colleague and co-founder of AD, Emil Kraepelin (Graeber et al., 1997).

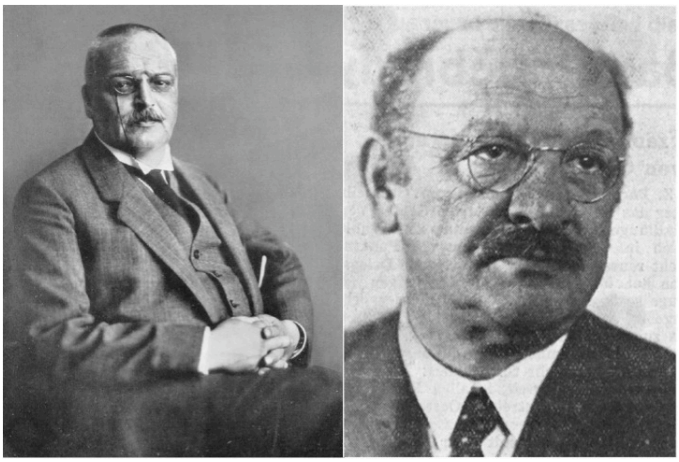

Figure 1 Alois Alzheimer (left) and Oskar Fischer (right)
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AD is the most common neurodegenerative disorder, diagnosed in people over 65 years of age (Brookmeyer et al., 1998), estimated to affect 35 million people worldwide and may quadruple by 2050, reports approximate every 69 seconds someone develops AD (Alzheimer'sAssociation, 2010; Brookmeyer et al., 2007). Early clinical symptoms of the disease are imperceptible and start with minor lapses in episodic memory. Later it develops into dementia and disorientation associated with a lack of interest in hobbies, word-finding difficulties, spatial disorientation, trouble with math, stereotyped manual tasks, disturbance of balance and other impairments (mild cognitive impairment). Disease severity progresses to global cognitive deficits, full disorientation, and profound memory impairment. In the last state, the patient is

commonly bed-ridden and dependent on permanent care. Self-portraits of an artist by name William Utermohlen depicts deleterious effect of the disease on him (Figure 2)

Figure 2 Self-portrayals by William Utermohlen. An artist who suffered from Alzheimer's disease painted a series of self-portraits during the progression of his illness. He died in 2007 leaving an expressive legacy, his paintings during his sufferings showed his descent into dementia. (Courtesy: Galerie Odille Boicos, Paris)

1.2 Neuropathological phenotype of Alzheimer's disease

Albeit the clinical symptoms, confirmation of AD requires post-mortem studies, especially the classical microscopic observation of lesions in hippocampal sections and the association cortices of the frontal, temporal and parietal lobes. Overall, multiple regions of the brain are affected as a result of the disease, including hippocampus, neocortex, amygdala, cholinergic system in the forebrain, and the monoaminergic systems of the brainstem's neuronal population (Arnold et al., 1991; Braak and Braak, 1991; Hyman et al., 1984; Whitehouse et al., 1982). The Aβ peptide was identified and sequenced from the meningeal blood vessels of AD patients in 1984 (Glenner and Wong, 1984), and the following year it was recognized as the primary component of the neuritic plaques in the aforementioned brain areas of AD patients (Masters et al., 1985; Scheff et al., 1990; Selkoe et al., 1986). Additionally, neuritic plaques are also present in the outer walls of parenchymal arterioles, small arteries, capillaries, and venules. Post-mortem studies of the brain suggest that the neuritic plaques and neurofibrillary tangles are the two principal neuropathological hallmarks of AD. Immunohistochemical studies show a decrease in the synaptic density in the frontal cortex of AD brain, suggesting reduction in active synaptogenesis (Scheff et al., 1990). Furthermore, loss of synaptic ending precedes the neuronal loss that leads to cognitive impairment (DeKosky and Scheff, 1990).

Neuritic plaques are found extracellular, proximally surrounded by activated microglia, reactive astrocytes, and dystrophic axons and dendrites (Pike et al., 1994; Rozemuller et al., 1986). Along with plaques, there are diffuse or pre-amyloid plaques

present in the same brain regions. Their occurrence is detected under light microscope in the brain lesions of AD patients and in cognitively normal, late middle-aged and elderly healthy individuals (Dickson et al., 1995; Price and Morris, 1999). Additionally, diffuse plaques generally lack amyloid fibrils and are not associated with dystrophic neurites or altered glia, suggesting these diffuse plaques may predate fibrillar neuritic plaques (Dickson, 1997).

Apart from neuritic plaques, the other diagnostic hallmarks of AD are the neurofibrillary tangles, visualized by Bielschowsky silver staining. Tangles are non-membrane bound masses of paired helical filaments (PHF), formed by a hyperphosphorylated form of the microtubule-associated protein, tau (Grundke-Iqbal et al., 1986).

Alternatively, Khachaturian proposed another hypothesis stating that disturbances in calcium homeostasis are a cause of neurodegeneration in AD (Khachaturian, 1994). Initially, this hypothesis was promulgated due to lack of enough supportive experimental evidence, while later reports backed the hypothesis (Mattson et al., 2000).

With respect to the neurotransmitter phenotype, degeneration of cholinergic neurons in the nucleus basalis of Meynert is responsible for cognitive impairment in AD (Davies and Maloney, 1976). Remarkable reduction of choline acetyltransferase (CAT) and glutamic acid decarboxylase activities (acetyl choline synthesizing enzymes) are found in AD brains (Perry et al., 1977). Davis along with other scientists further reported the involvement of GABAergic transmission in AD (Davies et al., 1998). In addition to the involvement of cholineric and GABAeric neurons, neurons using glutamate, somatostatin, corticotrophin releasing factor and serotonin also undergo degeneration in AD brains (Bennett et al., 1997; Moller, 1999). Due to the complexity and multiple neurotransmitter deficits involved in AD, apart from the treatment of cholinergic deficiency, no other factors seems to lead to serious therapeutic implications (Selkoe and Schenk, 2003).

1.3 Genetics of Alzheimer's disease

Advancements in molecular genetics have led to a better understanding of AD beyond the prominent age factor in sporadic forms. Mutations in at least one of three genes, encoding Presenilin 1, Presenilin 2 or APP, have been identified in familial AD (FAD), where cerebral levels of Aβ are elevated (Bertram and Tanzi, 2008). Numerous studies have been conducted to identify genetic links to the susceptibility of AD, since all the previously reported links (APP, PS1 and PS2) were related to rarely found early-onset AD. In the year 2002, Fagan and colleagues correlated Apolipoprotein E (ApoE) and Aβ to late onset Alzheimer's disease with some strong evidence (Fagan et al., 2002). ApoE is a plasma protein involved in the transportation of cholesterol and other lipids. There are three isoforms of ApoE (ApoE II, ApoE III and ApoE IV) found among humans. The ApoE4 isoform was reported to be involved in the development of late-onset AD. ApoE 4 is the only genetic risk factor for late onset AD (Bertram and Tanzi, 2008). Decreased clearance of the extracellular Aβ due to impaired function of neprylisin or insulin degrading enzyme (IDE) (Qiu et al., 1998), which is an Aβ degrading protease, is speculated to be the reason for the pathogenesis. Additionally, due to an increased β-secretase expression in the brain, and thereby increased cleavage by β-secretase, there will be an increase in Aβ production.

1.4 Cell and molecular biology of Alzheimer's disease

1.4.1 Amyloid precursor protein

The Amyloid precursor protein (APP) is a member of a family of evolutionarily conserved type I transmembrane glycoproteins. The APP gene is located on chromosome 21 (Goldgaber et al., 1987; Kang et al., 1987; Ponte et al., 1988; Robakis et al., 1987; Tanzi et al., 1987; Tanzi et al., 1988). APP occurs in three major isoforms, APP695, APP751, and APP770. Their molecular weight ranges between 110-140 kDa, depending on alternative splicing. Of these, APP695 is predominantly expressed in neurons, while the other two variants are expressed in neuronal and non-neuronal cells (Sisodia et al., 1993). APP751 and APP770 lack a 57-residue domain homologous to Kunitz serine protease inhibitors family (LeBlanc et al., 1991).

1.4.2 APP processing and secretase activities

Newly synthesized transmembrane APP is translocated into the endoplasmic reticulum (ER) and post-translationally modified through the secretory pathway. Post-translational modifications of APP include phosphorylation, tyrosine-sulphation and *N*- and *O*-linked glycosylations (Oltersdorf et al., 1990; Weidemann et al., 1989). The mature APP undergoes rapid degradation following the transportation via the cell surface, through an endocytic or a biosynthetic pathway. Only the immature APP carrying N-glycosylations undergoes degradation either in the ER or in the cis-Golgi (Cook et al., 1997; De Strooper et al., 1993; Haass et al., 1992; Hartmann et al., 1997; Kuentzel et al., 1993; Marambaud et al., 1997; Sambamurti et al., 1992; Shoji et al., 1992). Aβ peptides are cleavage products of APP, successively cleaved by β and γ secretase (aspartyl proteases). Apart from these two secretases, α-secretase cleavage in the Aβ domain of APP molecule prevents Aβ production (Esch et al., 1990; Sisodia et al., 1990). Thus, depending upon the secretase involved in APP cleavage, the pathway is termed either amyloidogenic or non-amyloidogenic.

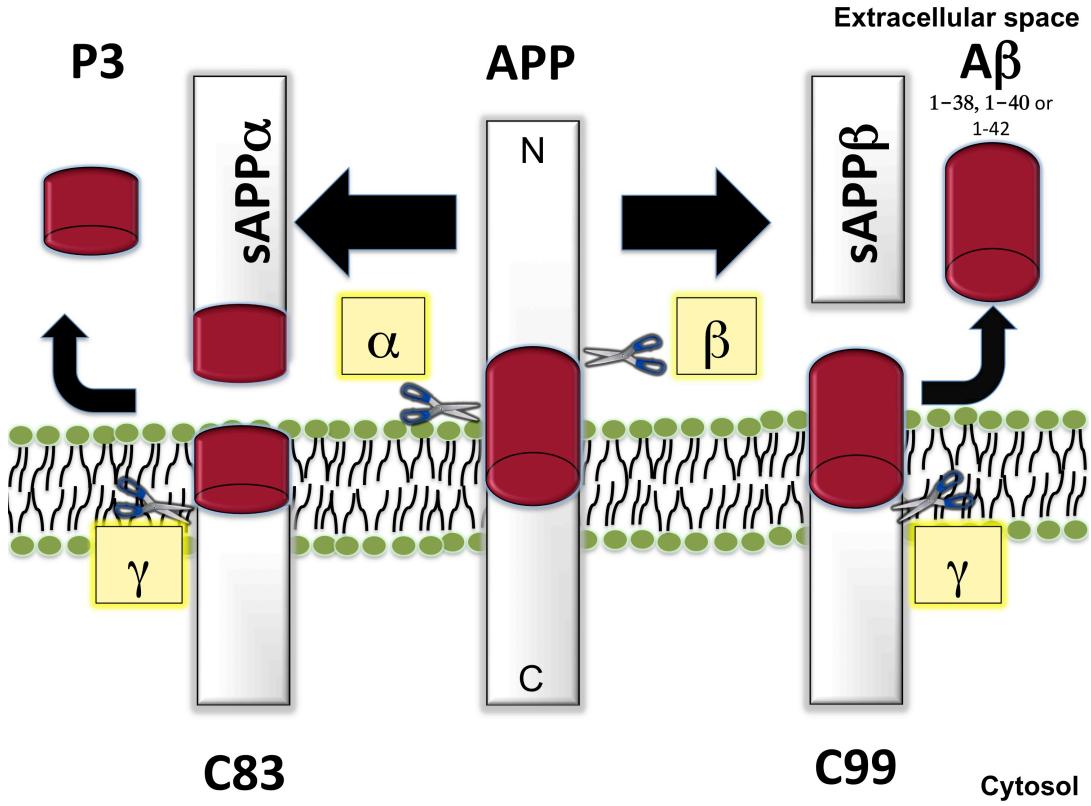

Figure 3 APP processing. APP processing involves proteolytic cleavage by α or β secretase and γ secretase. In the non-amyloidogenic pathway, α-secretase cleavage occurs in the middle of Aβ sequence and leads to the release of several soluble APP fragments. The amyloidogenic pathway is initiated by β-secretase followed by γ secretase releasing Aβ.

In the non-amyloidogenic pathway, α-secretase cleavage occurs within the N-terminal domain of APP to release a large ectodomain fragment of soluble APPα (sAPPα, 105-125 kDa) into the extracellular space and retention of C-terminal fragment (α-CTF or C83) in the membrane (Haass et al., 1993). α-secretase cleaves within the Aβ domain (Lys16-Leu17 bond) precluding the formation and deposition of intact Aβ peptide (Lammich et al., 1999). The secreted sAPPα has neuroprotective and memory enhancing effects (Furukawa et al., 1996; Meziane et al., 1998). α-CTF, the membrane bound product of α-secretase becomes substrate for γ-secretase cleavage resulting in the release of p3 fragment to the extracellular space and AICD (APP intracellular domain) into the cytosol (Esch et al., 1990; Haass et al., 1993; Haass et al., 1992).

In the amyloidogenic pathway, β-secretase cleavage occurs in the ectodomain of APP, releasing soluble APP-beta fraction (sAPPβ) into the extracellular space and leaving C-terminal fragment of 99 amino acids (β-CTF or C-99). Subsequently, γ-secretase cleaves the β-CTF to secrete Aβ into extracellular space and the APP intracellular domain (AICD) is released into the cytosol. AICD triggers a molecular cascade that is believed to be acting as apoptotic signal (Ozaki et al., 2006; Passer et al., 2000).

1.4.3 Secretases

1.4.3.1 α-secretase

Alpha secretase is a zinc metalloproteinase and member of the Disintegrin and Metalloprotease domain (ADAM) protein family, which are expressed on the cell surface and anchored within the cell membrane. Several members of adamalysin family proteins like tumor necrosis factor-α convertase (TACE) ADAM17, ADAM10 and ADAM9 show α-secretase activity (Furukawa et al., 1996; Mattson et al., 1999; Meziane et al., 1998).

1.4.3.2 β-secretase

β-secretase, also called as β-site APP cleaving enzyme (BACE1) or memapsin-2, is a type I trans-membrane protein selectively expressed in neurons and has a role in myelin sheath formation (Willem et al., 2006). BACE1 contains two active aspartate

protease motifs, which cleaves APP and determines the N-terminal end of Aβ (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999).

1.4.3.3 γ-secretase

γ-secretase has three cleavage sites releasing three different variants of Aβ peptide with variable lengths. γ-secretase cleaves APP following ε (position 49) and ζ-cleavages $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ are three major species of Aβ corresponding to the different cleavage sites.

1.4.4 Amyloid hypothesis and Alzheimer's disease

In 1838, Mathias Schleiden first coined the term amyloid to describe the amylaceous constituent of plants (Kyle, 2001). Amyloid is a long straight unbranched fibril with a diameter of ~100 Å. It is made up of parallel protofilaments having a cross β-sheet and can be stained using Congo red dye (Bennhold, 1922).

Until 1992, Aβ production was considered as a pathogenic event, but this view point was changed after reports showing the presence of Aβ in both CSF and plasma in healthy individuals. On the other hand, over-production of Aβ or increased ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$ was suspected to be the cause in early onset AD (Bentahir et al., 2006; Cai et al., 1993; Citron et al., 1992; Kumar-Singh et al., 2006; Rovelet-Lecrux et al., 2006; Suzuki et al., 1994).

Meanwhile, the amyloid hypothesis became one of the hot topics in the scientific community as well as highly controversial (Marx, 1992). The skepticism was due to the inconsistency of Aβ toxicity observed in some laboratories. In the next two years, Pike et al and Lorenzo reported that the neurotoxicity of amyloid beta requires a specific structure (Lorenzo and Yankner, 1994; Pike et al., 1993).

1.4.4.1 Evidences for Amyloid cascade hypothesis

There is mounting evidence supporting the role of Aβ as a causative agent in AD. AD-like neuropathology is observed in Down's syndrome (Trisomy of chromosome 21) caused by APP over-expression and increased Aβ production (Mann et al., 1984; Motte and Williams, 1989; Olson and Shaw, 1969). Supporting this observation, reports suggest that the duplication of the APP locus on chromosome 21 leads to early

onset of AD or cerebral amyloid angiopathy among some unrelated families (Rovelet-Lecrux et al., 2006). A mouse model with mutant human APP show increased extracellular Aβ accumulation and mimics AD pathology and behavioural changes (Ashe, 2005).

Mutations in the APP gene within or around the Aβ region which influence production or aggregation rate of Aβ, are found in patients with familial history of early-onset AD (Chartier-Harlin et al., 1991; Goate et al., 1991; Levy et al., 1990). Increased $A\beta_{1-42}$ to $A\beta_{1-40}$ ratio due to inherited mutations in the presenilin genes cause early and severe forms of AD (Bentahir et al., 2006; Kumar-Singh et al., 2006).

Fagan et al showed the ApoE affects the Aβ metabolism, deposition and clearance, and facilitates Aβ fibrillogenesis. Holtzman et al demonstrated that the murine apoE influences Aβ deposition using ApoE knockout mice (Holtzman et al., 1999). Furthermore, in a comparison study, where Aβ plaque burden was analyzed between APP transgenic mice with either of three different human ApoE isoforms (II, III or IV), it was shown that the ApoE isoform influences Aβ deposition. Transgenic mice with ApoE4 were reported to have higher levels of Aβ deposits in the brain when compared to the transgenic mice with ApoE2 or ApoE3 (Fagan et al., 2002). Additionally, *in vitro* studies show that the ApoE binding to Aβ induces Aβ fibrillation (Strittmatter et al., 1993). In summary, numerous reports have confirmed that the ApoE 4 allele is correlating to AD risk factor and ApoE 2 allele appears to be protective against AD (Corder et al., 1994; Corder et al., 1993; Mayeux et al., 1993; Strittmatter et al., 1993). Recently, it has been shown that ApoE4 impairs clearance of Aβ across the blood brain barrier (Deane et al., 2008).

In support of the Amyloid hypothesis, several groups reported that the synthetic form of Aβ (with different oligomerization strategies) show neurotoxic effects both *in vivo* and *in vitro* (Barghorn et al., 2005; Busciglio et al., 1995; Deshpande et al., 2006; Hartley et al., 1999; Hoshi et al., 2003; Kayed et al., 2003; Lambert et al., 1998; Pike et al., 1991). Injection of the synthetic peptides into either tau over-expressing transgenic mice, or into animals with human mutations of APP and tau, triggers tau hyperphoshorylation, which leads to tangle formation (Gotz et al., 2001; Lewis et al.,

2001; Oddo et al., 2003; Santacruz et al., 2005). Reports showing a connection between tau and Aβ further strengthened the amyloid cascade hypothesis (Rapoport et al., 2002).

In primary neuronal cultures, Aβ peptides have been reported to induce degeneration through one or more of the following pathways- apoptotic pathways (Loo et al., 1993); by induction of toxic levels of reactive oxygen species (Behl et al., 1994); triggering nitric oxide production through a NFκB-dependent mechanism (Hensley et al., 1994); activating the complement cascade pathway by binding to C1q (Rogers et al., 1992); or Aβ can activate the complement cascade without mediation of immunoglobulins.

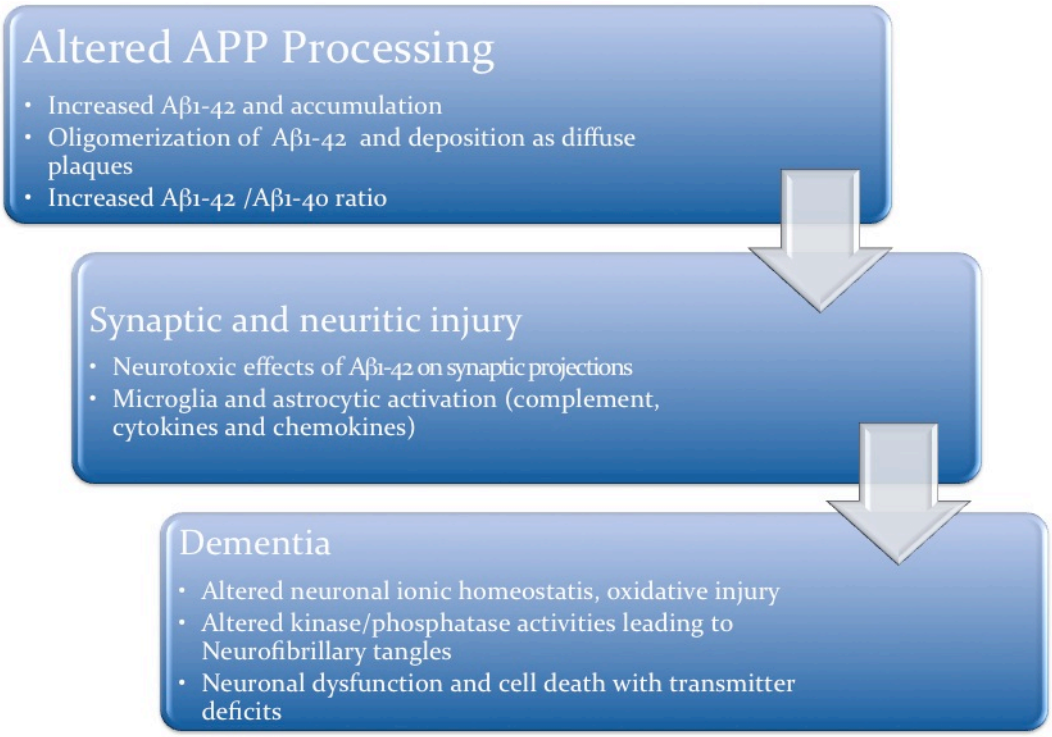

Figure 4 Amyloid cascade hypothesis: Accumulation of $A\beta_{1-42}$ that initiates a sequence of pathogenic events that are speculated to lead to AD.

1.4.4.2 Intracellular Aβ

Soon after the Aβ peptide was reported to be the main component of extracellular amyloid plaques, there were some studies claiming the presence of intracellular Aβ. Both AD and control groups were reported to have intracellular Aβ (Grundke-Iqbal et al., 1986).

1.4.4.3 Amyloid beta oligomers but not fibrils are toxic to neurons

Historically, Aβ fibrils have been considered to be primarily responsible for neuronal dysfunction and death, since fibrils were detectable in diseased brains and leading to neuronal dysfunction, dystrophy and synaptic loss (Geula et al., 1998; Grace and Busciglio, 2003; Pike et al., 1991). Buscigloio et al demonstrated that amyloid fibril formation alters the tau phosphorylation, which leads to loss of its microtubule binding capacity (Busciglio et al., 1995).

There are contradictory reports with respect to toxicity of amyloid plaques. A few reports dismissed the role of amyloid fibrils on AD, due to the lack of correlation between the plaques and AD pathogenesis (Dickson et al., 1995; Katzman, 1986; Terry et al., 1991). On the other hand some investigators supported the concept of amyloid fibrils being toxic (Howlett et al., 1995; Lorenzo and Yankner, 1994).

However, emerging evidence suggests that Aβ has the ability to form a diverse set of different assemblies, called oligomers, starting from dimers, trimers, tetramers, octamers, dodecamers (Aβ*56), all the way up to protofibrils (Barghorn et al., 2005; Kayed et al., 2003; Lesne et al., 2006; Shankar et al., 2008). In contrast to fibrils, these oligomeric forms of Aβ are non-fibrillar, water-soluble assemblies of Aβ following different aggregation pattern and cause neuronal death through different pathways (Walsh et al., 2002).

1.4.4.4 Aβ oligomers in human and transgenic mice brain

Biochemical analyses of AD brain samples indicate that the amount of non-fibrillar Aβ is associated with synaptic loss by impairing glutamanergic transmission, blocking long-term potentiation (LTP), and dementia (Kamenetz et al., 2003; Snyder et al., 2005; Walsh et al., 2002). Although, insoluble amyloid aggregates and fibrils are present in AD patients' brain, soluble oligomers of amyloid are reported to play a crucial role in AD pathogenesis. In particular, extracellular accumulation of $A\beta_{1-42}$, which is more prone to oligomerize compared to $A\beta_{1-40}$, affects the synaptic plasticity, induces a loss of synaptic dendritic spines, and adds to the severity of cognitive impairment (Hsieh et al., 2006; Jarrett et al., 1993; Lue et al., 1999; McLean et al., 1999; Wang et al., 1999). Despite of assays that could measure soluble Aβ, the

aggregation state of Aβ could not be identified (Funato et al., 1999; Morishima-Kawashima and Ihara, 1998; Stenh et al., 2005).

Samples from frontal cortex and putamen revealed the presence of monomeric, dimeric and trimeric species of Aβ (McLean et al., 1999). Human CSF samples and brain extracts showed the presence of SDS-stable low molecular weight oligomers but no higher oligomers, which are non-covalently associated with dimeric and trimeric species of Aβ (Enya et al., 1999; Funato et al., 1999; McLean et al., 1999; Roher et al., 1996; Vigo-Pelfrey et al., 1993).

In the year 2003, Kayed and colleagues produced a synthetic Aβ peptide mimicking spherical structure and produced antibodies, which they reported as being "conformation specific" antibodies (Kayed et al., 2003). The authors could show that the toxicity exerted by oligomers on human neuroblastoma cells (SH-SY5Y cells) could be decreased after the treatment with oligomer specific antibodies.

On the other hand, Lesne and colleagues observed that there is no impairment of spatial memory in Tg2576 transgenic APP mice in the presence of monomers, trimers, or hexamers of Aβ during the first 6 months. Later, the appearance of SDS-stable nonamers and dodecamers directly correlate with the changes in spatial memory (Lesne et al., 2006). Intraventricular injection of dodecamer Aβ purified from diseased brain affect the spatial memory remarkably. The authors addressed these dodecamers as "Aβ*56" which may be considered as representative of Aβ derived diffusible ligands (ADDLs) derived from brain. In contrast, Lesne and other two groups pointed out the lack of correlation between dodecamers and other forms on memory, and synaptic form and function (Dineley et al., 2002; Jacobsen et al., 2006; Lesne et al., 2006). Recently, Shankar et al showed that dimeric Aβ, isolated directly from AD patient brains, can act as potential toxic species, impairing LTP and affecting learned memory (Shankar et al., 2008).

1.4.4.5 Synthetic Aβ protofibrils, ADDLs and globulomers

Snyder and colleagues first reported the presence of Aβ species apart from fibrils using synthetic peptides (Snyder et al., 1994). In the same year, Oda et al showed that

Aβ and Clusterin (Apo J) mixtures give rise to small toxic assemblies other than fibrils and reported for the first time the toxic effect of synthetic Aβ *in vitro* (Oda et al., 1994; Oda et al., 1995).

1.4.4.5.1 Aβ Protofibrils

Pioneering studies from Walsh and Teplow showed two distinct derivatives formed by $A\beta_{1-40}$ and $A\beta_{1-42}$ called protofibrils. Protofibrils precedes fibril formation and has similar structural features (Walsh et al., 1997), whose formation is dependent on pH, concentration and ionic strength (Harper et al., 1997). These 'metastable intermediate' protofibrils form fibrils and can dissociate into low molecular weight species (Harper et al., 1999; Walsh et al., 1999). Treating primary neurons with protofibrils demonstrated their toxicity, induced excitatory post-synaptic currents, and large membrane depolarization effects (Hartley et al., 1999).

1.4.4.5.2 Aβ-derived diffusible ligands (ADDLs)

In 1998, Lambert and Klein reported a small globular and diffusible form of $A\beta_{1-42}$ called ADDLs that assembled when fibril formation is inhibited by synthetic peptides. ADDLs are derivatives of synthetic Aβ which appear before the protofibril formation and it is reported that they block LTP and cause neuronal death at very low concentrations (Lambert et al., 1998; Wang et al., 2002). Following these reports, there is quite a number of publications supporting that ADDL preparations could be toxic in both *in vitro* and *in vivo*.

1.4.4.5.3 Globulomer-like ADDLs

There are several ADDLs like structures available today, of which a few are more noteworthy: Kayed et al produced Aβ with unique conformation using gold particles. These oligomers may start from tetramer to higher oligomers without fibril formation and share the same conformation with other 'Amyloid-like' synthetic peptides and proteins (Kayed et al., 2003). Lesne and colleagues showed Aβ*56, a dodecamer produced under *in vivo* conditions which could affect memory in transgenic animals. In the year 2005, Barghorn et al reported a synthetic peptide forming a stable, homogenous globular structure, which they addressed as 'globular amyloid oligomer – globulomer' that affects memory and LTP in transgenic animals (Barghorn et al., 2005).

1.4.4.5.4 Aβ oligomers of cellular origin

Similar to human and mouse brain extracts, SDS-stable low molecular weight oligomers have been detected in conditioned cell culture medium by over expressing hAPP (Morishima-Kawashima and Ihara, 1998; Podlisny et al., 1995; Townsend et al., 2006; Walsh et al., 2000). Walsh et al., purified the low molecular weight oligomers and showed that these oligomers are not only stable to SDS but also to IDE that degrade monomeric Aβ. Further, authors showed cell-derived oligomers inhibit the maintenance of hippocampal LTP, decrease synaptic activity, which is in line with severely affected AD. This effect did not occur on using either Aβ monomers or any larger aggregates (Walsh et al., 2005). These observations were supported by Cleary et al showing natural oligomers could disrupt cognitive function (Cleary et al., 2005). Furthermore, naturally secreted Aβ trimers were found to be neurotoxic and affects some specific forms of hippocampal LTP (Townsend et al., 2006).

1.4.5 Neuroinflammation in AD

The human brain is not an immunologically isolated organ. Despite the earlier dismissals claiming immunological features of AD brain as an artefact, mounting evidence support AD as a neuroinflammatory disease (Akiyama et al., 2000). Neurons and neurites in the AD brain, insoluble Aβ deposits and NFTs act as stimuli for inflammation. Microglial cells, the principal immune cell in the brain, are abundant around the plaque deposits exhibiting reactive or activated phenotype in AD brain (Wisniewski HM, 1973).

1.4.5.1 Cytokines

Neuroinflammation with elevated cytokine levels is one of the pathological features found in the AD brain. In AD brains, activated microglial cells and reactive astrocytes proximally surround extracellular neuritic plaques (Wisniewski HM, 1973). These cells release many neuroinflammatory mediators like pro-inflammatory cytokines (Griffin et al., 1989), chemokines, proteases, protease inhibitors, adhesion molecules, free radicals, and activate the complement systems. The involvement of inflammatory mediators in AD brains was first reported with respect to upregulation of the cytokines

interleukin-1 (IL-1) and S100 (Griffin et al., 1989). IL-1 is responsible for initiating dystrophic neurite formation in diffuse Aβ deposits (Griffin et al., 1995). IL-6 was found to be elevated around senile plaques in the cortical and hippocampus regions of AD brains (Bauer et al., 1991; Strauss et al., 1992). Further, the involvement of IL-8, tumor necrosis factor-alpha (TNF-alpha) and TGF-β were found in the pathogenesis of Alzheimer's disease (Akiyama et al., 2000).

On the other hand, soluble APP-alpha (sAPPα) has been reported to be neuroprotective. A recent review suggests that there is a role of cytokine suppression in regulating the α- secretase function (Sastre et al., 2008).

1.5 Alzheimer's disease therapy

Average human life expectancy increased dramatically with scientific/medical advancements. Increased number of age-related diseases like AD needs urgent and efficient therapeutic strategies to influence the disease progression. Albeit the current understanding of AD is still incomplete, there are rational efforts that could bring some potential diagnostic tools and therapeutic agents to halt and/or reverse the disease progression. Pharmaceutical companies are in a stiff competition to develop new therapeutics for AD, but the recent failures in clinical phases forebodes the chances of many drug candidates remains murky.

1.5.1 Drugs to treat Alzheimer's disease

Existing medicaments offer symptomatic relief to AD patients either by inhibiting neurotransmitters or modulating/blocking potential receptors in the brain (Table 1), however, does not help to reverse or halt disease progression. Considering the role of Aβ aggregation in AD, Aβ is without dispute, a valid molecular target to treat the disease. Two strategic approaches have been intensively implemented, (i) either to inhibit the production of Aβ or, (ii) to prevent of aggregation/formation and elimination of toxic forms of Aβ from the system. Despite decades of hard-work very few drugs targeting either of the two approaches entered the clinical phases. But as of now, there are no drugs available in the market with any of those two curative approaches. Recently, a gamma secretase inhibitor (Semagacestat) was dropped out of the clinical phase III due to adverse effects (Lilly, 2010).

Table 1 List of drugs currently used to treat AD

Brand Name/Generic Name	Mechanism	Year of approval
Namenda/Memantine	NMDA antagonist	2003
Razadyne/Galantamine	AChEI, NRM	2001
Exelon/Rivastigmine	AChEI, BuChEI	1996
Aricept/Donepezil	AChEI	1996
Cognex/Tacrine	AChEI, BuChEI	1993

AChEI = Acetylcholinesterase inhibition
BuChEI = Butyrylcholinesterase inhibition
NRM = Nicotinicreceptor modulator
NMDA = N-methyl d-aspartate

1.5.2 Immunotherapy

Immunization strategies, both active and passive, showed reduction of the Aβ plaque load and helped the restoration of cognitive deficits in transgenic mice (Bacskai et al., 2002; Dodart et al., 2002; Gelinas et al., 2004; Klyubin et al., 2005). Several supportive evidences suggest immunotherapy as one of the most promising therapeutic strategies to treat AD. As a straightforward vaccination approach, Schenk and colleagues immunized APP transgenic mice with the Aβ aggregated form of $A\beta_{1-42}$ (Schenk et al., 1999). This strategy later paved the way for a passive immunization approach, where, for the first time, Dodart et al and Kotilinek treated APP transgenic mice with amyloid specific antibodies (Dodart et al., 2002; Kotilinek et al., 2002).

1.5.2.1 Active immunization

Direct immunization with synthetic Aβ aggregates was the first immunotherapeutic approach with respect to AD (Schenk et al., 1999). Strikingly, the AD pathology was reversed among the treated transgenic mice compared to the control group. The mode of action involves activation of microglia and stimulation of T and B-cell lymphocytes. Unlike the amyloid hypothesis, this immunotherapy approach was easily reproduced by other groups, who published similar results with some proposals on the underlying mode of action (Janus et al., 2000; Morgan et al., 2000). Short fragments conjugated with carrier protein were also used in active immunization, which triggers

B-cells to produce antibodies and stimulates T helper cells, leading to B-cell maturation that is mediated by cytokines (Sigurdsson et al., 2001). This approach minimizes the risk of T-cell response to Aβ. These studies suggested that the entire Aβ peptide was not needed to treat AD.

In 2002, Elan pharmaceuticals and Wyeth Corporation jointly conducted a phase IIa clinical trial with AN-1792 (pre-aggregated synthetic $A\beta_{1-42}$) with QS-21 adjuvant. The trial was suspended due to adverse response to the peptide vaccine. A small number of patients (18 out of 300), involved in the trial had symptoms resembling meningoencephalitis and microhemorrages (Bowers and Federoff, 2002; Munch and Robinson, 2002; Orgogozo et al., 2003). According to the report, the patients had less number of plaques compared to AD controls in the neocortex and reactive microglia around Aβ plaques (Ferrer et al., 2004; Nicoll et al., 2003). If meningoencephalitis and haemorrhages could be avoided, immunotherapy may be a potential approach to treat AD. As an aftermath effect, researchers were less interested to carry out with active immunization and passive immunization appeared as a potential and safer alternative.

1.5.2.2 Passive immunization

In the year 2000, Elan pharmaceuticals reported the first passive immunotherapy approach in the AD field. Bard and colleagues administered N-terminal specific ($A\beta_{1-15}$ and $A\beta_{1-16}$) monoclonal antibodies (3D6 and 10D5) intraperitoneally into PDAPP (human Platelet derived growth factor driven-human APP transgenic) mice for six months on a weekly basis (Bard et al., 2000). The results showed that the antibodies crossed the blood brain barrier (BBB) and lead to significant reduction of plaques both in the cortex and hippocampus. Estimation of the amount of IgG entering the brain showed that 0.11% of injected IgG enters the brain within 1 hour after intraperitoneal administration, and remains in the brain for 72 hours (Banks et al., 2002). The disruption of amyloid plaques and clearance is carried out through Fc mediated phagocytosis by microglia (Bacskai et al., 2002). Bard et al and Bacskai et al, used N-terminal specific antibodies, which can bind to the fibrils of amyloid that has protruding N-terminal sequence.

DeMattos et al treated the identical PDAPP mice using mid-terminal specific ($A\beta_{13-28}$) m266 antibodies and showed a reduction of Aβ deposition in the brain without binding

to the plaques (DeMattos et al., 2001). It is important to notice that the mechanism DeMattos proposed was due to the peripheral action of the antibodies, but not due to their entry into the brain. Intravenous injection of m266 into PDAPP mice resulted in 1000 fold increased plasma levels of Aβ compared to the PBS treated group. They used m266 as a 'capture antibody', which binds to the mid-term region of Aβ, making it inaccessible for other endogenous proteins to bind and facilitates Aβ clearance in the liver. This mechanism of Aβ clearance by immunotherapy is also called the "peripheral sink hypothesis". As per the peripheral sink hypothesis, the main role of these antibodies is to shift the equilibrium between soluble and aggregated Aβ assemblies towards soluble forms which further facilitates Aβ clearance. Similarly, the peripheral sink could also be observed with molecules like curcumin and phenolics, which can cross the BBB. These molecules bind to Aβ oligomers and fibrils to destabilize the complex leading to disaggregation of Aβ aggregates both in vitro and in vivo (Yang et al., 2005).

In the year 2002, the same group treated PDAPP mice with the same antibodies (m266), and reported a reversal of memory deficits within one day after injection (Dodart et al., 2002). The improvement of cognition was found with unaltered Aβ plaque burden in the brain. Authors hypothesized the effect may be due to the removal of soluble Aβ fractions from the brain.

1.5.2.2.1 Passive immunization with naturally occurring antibodies

For the past thirty years, intravenous immunoglobulins (IVIG) have been used to treat some diseases related to immunological background like autoimmune disease and immunodeficiency. The FDA has approved the treatment for primary immunodeficiencies, Kawasaki syndrome, B-cell chronic lymphocyctic leukemia (CLL), and bone marrow transplantation.

Previously our group and others reported that the naturally occurring autoantibodies against Aβ (NAbs-Aβ) are reduced in AD patients, which could interfere with fibril formation and reduce neurotoxicity (Du et al., 2001; Du et al., 2003; Weksler et al., 2002). Interestingly, IVIG preparations that are available have been shown to contain these NAbs-Aβ (Dodel et al., 2002). Very recently we have reported that the differences among IVIG preparation and storage conditions influence the amount of

NAbs-Aβ and to some extent differs upon their specificity towards $A\beta_{1-40}$ and $A\beta_{1-42}$ (Balakrishnan et al., 2010).

These autoantibodies are both fibril-specific and specific to toxic conformations of Aβ (O'Nuallain et al., 2008). Klaver and colleagues recently reported that some IVIG preparations have more monomer specific antibodies compared to oligomer specific antibodies (Klaver et al., 2009) and may also affect plaque burden and induce activation of microglia without increasing microhaemorrhages (Kellner et al., 2009).

Three pilot studies have been conducted to investigate the effect of IVIG in AD patients. The results showed that 6 months of treatment decreased Aβ levels in the CSF and increased plasma levels of Aβ (Dodel et al., 2004; Relkin et al., 2008). Based on the current evidence, NAbs-Aβ, which are part of the IVIG preparations, may serve as a potential treatment option for AD (Britschgi et al., 2009; Solomon, 2007).

The epitope region of NAbs-Aβ lies in the membrane bound C-terminal region of the Aβ (Balakrishnan et al., 2010). The rationale using monoclonal antibodies directed against Aβ protruding out of the membrane may be considered as an inappropriate target. This is because the physiological role of Aβ or the cleavage products are not completely understood yet. Considering this, NAbs-Aβ isolated from FDA approved IVIG preparations might be seen as a safer therapy for AD.

1.5.3 Anti-inflammatory drug therapy

In the year 1990, McGeer *et al* reported that the long-term treatment with anti-inflammatory drugs for rheumatoid arthritis patients showed signs of decreased dementia (McGeer et al., 1990). After this finding, there were reports that showed that different Non-steroidal anti-inflammatory drugs (NSAIDs) could modulate the secretase activity, Aβ levels, and also improved cognition after in animal models.

1.6 Potential targets to treat Alzheimer's disease

To summarise, since the Aβ peptide was sequenced in the year 1992, the century long senile plaque and dementia story faced a rollercoaster ride in the past two decades. These very short hydrophobic Aβ peptides have the potential to aggregate and form a

macroscopic fibrillar structures, these fibrillar Aβ were initially believed to be the key cause for AD. Several studies clearly show that the real causative agent of AD may range from a dimeric structure to soluble oligomers, protofibrils, insoluble higher aggregates to deposited fibrillar Aβ.

Due to the highly dynamic aggregation pattern of Aβ, lack of research techniques to identify the real culprit that cause AD and thereby the right target to treat AD remains a concern. Likewise, with the current knowledge of the disease, though it is inadequate, there should be a rational and safer approach for diagnosis and treatment.

With respect to the amyloid hypothesis, the possible molecular targets to treat AD could be, by influencing the aggregation rate or helping in disaggregation of toxic species, to shift the equilibrium from toxic species to soluble fractions that can be cleared and/or those that could bind to specific toxic Aβ forms and reduce receptor-mediated phagocytosis. Additionally, targeting the machinery involved in the Aβ production, which involves modulators and/or inhibitors of the secretases, is another possible approach to treat the disease.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Name	Supplier
1,1,1,3,3,3-Hexafluor-2-Propanol	Aldrich
Acetic Acid, Glacial 99+%	Sigma
Bis-Tris	Sigma
D-MEM	Gibco
Di-Potassium hydrogenphosphate trihydrate	Applichem
Di-Sodiumhydrogen phosphate-Dehydrate	Fluka
Di-Sodiumhydrogen phosphate* 2 H2O	Roth
DMSO	Sigma
Ethanol Absolute	Merck
Formaldehyde, 37 wt %	Sigma
Formic acid, 88%	Aldrich
Glycine	Roth
Hydrochloric acid 1N	Sigma
2 X LDS sample buffer	Invitrogen
MemCode™	Pierce
Methanol	Roth
Potassium Chloride	Fluka
Potassium dihyrogen phosphate	Merck
Precast 10-20% SDS PAGE gels	Invitrogen
Roti® Block	CarlRoth
SilverXpress staining kit	Invitrogen
Sodium Acetate- Trihydrate	Aldrich
Sodium bicarbonate	Roth
Sodium Carbonate	Aldrich
Sodium Chloride	Roth
Sodium dihyrogen phosphate Hydrate	Roth
Sodium Dodecyl Sulphate (SDS)	Sigma

Sodium Hydroxide pellets	Acros
Sodium phosphate* 7 H2O	Sigma
SulfoLink gel (UltraLink	Pierce
Sulphuric acid 99.99%	Aldrich
Supersignal West Dura Substrate	Pierce
Thioflavin T	Sigma Aldrich
Triton X-100	Roth
Trizma™ Hydrochloride	Roth

2.1.2 Equipments and devices

Name	Supplier
BioRad Mini-PROTEAN 3 Cell	Bio-Rad Laboratories, Richmond CA, USA
BIOTRAK II PLATE WASHER(C)4LN	GE Healthcare, Chalfont St. Giles, England
CO2-Inkubator CB210	Binder, Tuttlingen, Germany
Densiometer GS-800	Bio-Rad Laboratories, Richmond CA, USA
Eclipse80i Durchlichtmikroskop	Nikon GmbH, Langen, Germany
Elektrophoresekammer E-H3	Febikon GmbH, Wermelskirchen, Germany
Filmentwickler Optimax Typ TR	MS-Laborgeräte, Heidelberg, Germany
Fluoreszensmikrokop	Nikon GmbH, Langen, Germany
Fluoreszensmikrokop TE2000	Nikon GmbH, Langen, Germany
Geldryer Model 583	Bio-Rad Laboratories, Richmond CA, USA
Kippschüttler Rocky® 3D	Fröbel Labortechnik, Lindau, Germany
Laborwaage PCB	Kern, Balingen-Frommern, Germany
Magnet Dispergierer S	Heidolph, Kelheim, Germany
Magnetrührer Hei-Tec	VWR International GmbH, Darmstadt, Germany
Mikrotiterplatten-Photometer Infinite M 200	Tecan, Männedorf, Switzerland

Mikrozentrifuge 5415D	Eppendorf, Hamburg, Germany
Mikrozentrifuge Galaxy 16DH	VWR International GmbH, Darmstadt, Germany
Mini-Protean Tetra Cell	Bio-Rad Laboratories, Richmond CA, USA
MKR13 KühlThermoMixer	HLC BioTech, Bovenden, Germany
pH-Meter MP220	Mettler Toledo, Gießen, Germany
PowerPac Universal Power Supply	Bio-Rad Laboratories, Richmond CA, USA
Präzisionswaage BE BK	Satorius, Göttingen, Germany
Reagenzglasschüttler	VWR International GmbH, Darmstadt, Germany
Rotationsmikrotom Microm HM 355 S	Thermo Fisher Scientific, Rockford IL, USA
Rotor für 15 u. 50 ml Röhrchen, Nr. 3046	Heraeus, Hanau, Germany
Rotor für Reaktionsgefäße, Nr. 3332	Heraeus, Hanau, Germany
Sicherheitswerkbank HERAsafe/KS12	Heraeus, Hanau, Germany
Tischzentrifuge Biofuge Stratos	Heraeus, Hanau, Germany
Tischzentrifuge Megafuge 1.0 R	Bio-Rad Laboratories, Richmond CA, USA
Trans-Blot SD Semi-Dry Transfer cell	Nikon GmbH, Langen, Germany
Universal-Microscope ELWD 0.3	PeqLab, Erlangen, Germany
UV/Vis-Spectrophotometer NanoDrop ND-1000	Thermo Fisher Scientific, Rockford IL, USA
Vakuumzentrifuge Speed Vac RC 1010	UVP, Upland CA, USA
White Light Transiliminator	Invitrogen, Carlsbad CA, USA
Xcell II Blot Module	Bio-Rad Laboratories, Richmond CA, USA

2.1.3 Antibodies

2.1.3.1 Monoclonal antibodies

Name	Epitope	Supplier
3D6	Aβ 1-5	Elan
m266	Aβ 17-24	Lilly
A11	Spherical Aβ oligomers	Kayed
4G8	Aβ 17-28	Calbiochem
6E10	Aβ 1-16	Calbiochem, Covance
5C3	C-terminal Aβ 1-40, but not Aβ 1-42	Calbiochem

2.1.3.2 Secondary antibodies

Name	Supplier
Goat-anti-human antibodies HRP tagged	Calbiochem
Goat-anti-mouse antibodies HRP tagged	Calbiochem
Goat-anti-rabbit antibodies HRP tagged	Calbiochem

2.1.4 Polyclonal antibodies

Name	Supplier
Intravenous immunoglobulins	Octagam/Octapharma, Germany
Intravenous immunoglobulins	Gammagard/Baxter, Germany
Intravenous immunoglobulins	Kiovig/Baxter, Germany
Intravenous immunoglobulins	Gammunex/Bayer, Germany
Intravenous immunoglobulins	Intratect/Biotest Pharma, Germany
Intravenous immunoglobulins	Pentaglobin/Biotest Pharma, Germany
Intravenous immunoglobulins	Sandoglobin/CSL Behring, Germany

2.1.5 Cell culture supplements

Name	Supplier
DMEM	Invitrogen, Karlsruhe, Germany
F12 Medium	Invitrogen, Karlsruhe, Germany
Fetal Bovine Serum	Invitrogen, Karlsruhe, Germany
Penicillin	Invitrogen, Karlsruhe, Germany
Streptomycin	Invitrogen, Karlsruhe, Germany
Trypsin EDTA	Invitrogen, Karlsruhe, Germany

2.1.6 Buffers and standard solutions

All reagent solutions were prepared using double distilled and deionized water from a deionization system (Millipore Q) if not indicated otherwise.

PBS – phosphate buffered saline, pH 7.4

- 150 mM Sodium chloride
- 2.5 M Potassium chloride
- 10 mM Sodium phosphate
- 1.5 M Potassium phosphate dibasic
- ELISA washing buffer

1 X PBS tween (0.05% Tween 20)

Well coating buffer

- 0.1 M Sodium-bicarbonate buffer pH 9.6

Immunoprecipitation buffer (TBS – Tris buffered saline pH 7.5)

- 50 mM Tris
- 150 mM Sodium chloride
- 1 mM EDTA

2.2 Methods

2.2.1 Amyloid oligomer preparation

2.2.1.1 Amyloid beta monomers

$A\beta_{1-42}$ tends to aggregate faster than $A\beta_{1-40}$. In order to avoid aggregation, freeze-thaw procedure should be avoided as much as possible. DMSO keeps the peptide in monomeric state, for experiments where one needs monomeric forms of Aβ, DMSO is the ideal solution to resuspend the peptide. DMSO treated peptides should not be used for Dot blots. HFIP treatment is another alternative, if the peptides are not used immediately for the cell culture experiments.

2.2.1.2 Amyloid beta dimer enrichment

To get enriched dimers, N-terminal cysteine-containing $A\beta_{1-40}$ should be used. Due to rapid aggregation ration cysteine tagging could not be used for $A\beta_{1-42}$ and therefore $A\beta_{1-42}$ dimers are not easily prepared. Lyophilized peptides should be dissolved in 1 X PBS for dimerization followed by the monomerization (to avoid solubility issues, peptides were incubated for 15 minutes (mins) in an ultrasound water bath with 65°C). During the preparation turbidity is normal, but precipitation or larger aggregates was avoided. Subsequently the peptide solution was centrifuged at 12,000 rpm for 10 mins at +4°C to get rid of the precipitate. The resulting supernatant was the quantified using Nano drop (2.2.8.2). The peptide solution was incubated at 37°C for 3 hours on a heated shaking block (900 r.p.m). 2 µl of ammonia solution was added to stop the oligomerization by bringing the pH to approximately 10. Alternatively, incubated peptide solutions were used for the experiments or immediately transferred to -80°C freezer for storage.

2.2.1.3 Preparation of mutant Aβ trimer

Trimer preparation were exclusively done by two point mutations at the C-terminal end of the peptide $A\beta_{1-40;\ G29,33I}$. Resuspending the peptide in Sodium carbonate buffer of pH 9.6 brings trimeric structure which is stable for at least 2 weeks at +4°C, dissolving the peptide in other buffers will make them aggregated.

2.2.1.4 Preparation of toxic Aβ oligomers

Preparation of toxic Aβ oligomers were performed as described by Kayed *et al* (Kayed et al., 2003). Briefly, 200 µg of $A\beta_{1-42}$ were dissolved in 200µl of HFIP. Throughout the incubation steps, HFIP and HFIP containing solutions were handled under the ventilated hood. The peptide dissolved in HFIP was evaporated at room temperature (approx. 5-6 hours) or carefully lyophilized without drying the peptide. The peptide was then dissolved to obtain 1 mg/ml concentration of water + HFIP (25% v/v), and incubated for 2 days at 37°C with Teflon-coated magnetic stirrer with 900 rpm. The evaporated volume was then made up using MQ water and used for toxicity experiments immediately.

Samples were prepared with all possible combinations of parameters mentioned above. Identical samples were prepared in the same vial to avoid pippetting error, evaporation of sample due to small volumes etc., Peptides incubated for particular time points were stored by freezing in -80°C or leaving them at +4°C after addition of Ammonia.

2.2.2 SDS-Polyacrylamide gel electrophoresis (PAGE) and blotting

Ready to use pre-cast gels were set in electrophoresis cell chamber (BioRad), filled with respective running buffers for 4-12% Bis-Tris gels or 10-20% Tricine gells. The power supply Power Pac (BioRad) was set to constant voltage at 125 V for 55 mins or until the sample dye reached the bottom of the gel (according to manufacturers' instructions).

2.2.3 Western blotting

Nitrocellulose transfer membrane was washed with distilled water and soaked in blotting buffer. Gels were carefully removed from the plates. The transfer membrane was placed on the gel between two filter papers soaked with blotting buffer and three sponges on both sides and placed on the transfer membrane. The sandwich was placed in the blotting chamber filled with blotting buffer and with an ice cube block. The proteins were transferred from the gel to the transfer membrane for 2 hours at constant 400 mA.

2.2.3.1 Blocking procedure

The transfer membrane was washed twice with PBS, MemCode stain was added and incubated for 2 mins followed by washing thrice with distilled water before adding

destain solution. This procedure is to check the transfer of proteins. Later stain erase solution was added and incubated until the stain was removed. The membrane was then boiled for 10 mins in PBS before blocking to increase the signal. The membrane was blocked for 1 hour at room temperature or overnight at +4°C in Superblock blocking buffer.

2.2.3.2 Primary antibody incubation

Monoclonal antibodies were diluted 1:4000 dilution or as recommended by the providers in Roti-block blocking buffer. Transfer membranes were incubated for 2 hour at room temperature or at +4°C overnight.

2.2.3.3 Secondary antibody incubation

Followed by the incubation with the primary antibody, the membrane was washed 4 times with PBS + 0.5% Tween for 10 mins each wash. After washing the membrane, it was incubated with the appropriate horseradish peroxidase (HRP)-conjugated second antibody at 1: 20,000 dilution in PBS + 0.5% Tween for 1 hour at room temperature.

2.2.3.4 Detection

Proteins were visualized by enhanced chemiluminescence (super signal) following the manufacturer's instructions. The signals were exposed on X-ray films for an appropriate time and the films were developed using a film developer machine.

2.2.4 Dot blotting

Dot blotting has advantage of detecting conformational epitopes and therefore an effective technique to study conformational antibodies. 2 µl of Aβ peptides (1µg/ml) dissolved in the respective buffer according the oligomerization protocol were directly dotted onto a nitrocellulose membrane. The membranes were air-dried and incubated for 1 h at room temperature in blocking solution (Roti block, blocking solution), followed by an incubation step with anti-Aβ specific antibodies (1:250,000 dilution). Membranes were washed intensively four times with blocking solution and then incubated for 2 h in HRP-tagged antibodies directed at specific species of the primary antibody in blocking solution. Super Signal substrate was added and incubated for 3 mins followed by washing with deionized water and signal was developed.

2.2.5 Immunoprecipitation

Similar to Dot blotting, Immunoprecipitation has the advantage of allowing conformational antigen-antibody interaction. 5µg of antibodies and 3µg of specific Aβ enriched species in 100 µL IP buffer (TBS buffer pH 7.2) were incubated at +4°C overnight with end-over-end rotation. 100 µl of Protein G column slurry was washed twice with IP buffer followed by one wash with 1 X Roti block blocking buffer and incubated at +4°C overnight end-over-end. Blocking protein G slurry was then washed with IP buffer prior to the addition of antigen-antibody complex. This complex was incubated at room temperature for 1 hour with end-to-end rotation. After the incubation, the complex was washed 5 times with IP buffer followed by centrifugation at 13,000 g. 2 X LDS sample buffer and 1 X reducing agent was added to the slurry and boiled for 10 minutes with occasional vortex. Samples were then loaded over 10-20% Tricine gel (Invitrogen) and electrophoresed for 90 mins.

The gel was then transferred over Nitrocellulose membrane (0.2µM) for 2 hours at 200 mA. Protein bands over the transferred membrane were then visualized by memcode (Invitrogen). Membrane was then boiled for 5 mins with 1 X PBS followed by 1 X Roti block blocking buffer for 1 hour. 2 µg of anti- Aβ antibodies were added and incubated with constant shaking at 4°C, overnight. Membrane was washed 5 times with 1 X PBS with 0.05% Tween prior to the addition of secondary antibody tagged with HRP and incubated for 1 hour at room temperature. Super Signal substrate (Pierce, Germany) was added and incubated for 3 mins followed by washing with deionized water and signal was developed.

2.2.6 Silver staining

Silver staining provides an easy method to detect protein staining with high sensitivity with the lowest background, especially Aβ oligomers. Electrophoresed gels were stained using SilverXpress staining kit according to manufacturer's instructions.

2.2.7 ELISA

2.2.7.1 Antibodies quantification ELISA

96 wells high binding plates were coated with Aβ peptides and incubated for 2 hours at 37°C in CO2 incubator (5% CO2). Plates were blocked with a commercial blocking

buffer; incubated overnight. Standard antibodies and diluted serum samples were added to the plates after 4 washing cycles after blocking step and incubated for 4 hours at 37°C in CO2 incubator. Plates were washed with 4 washing cycles with ELISA plate washer with washing buffer.

HRP tagged anti-human secondary antibody (1:4000 dilution in Superblock) was used as detection antibody, plates were washed thoroughly (5 washing cycles); TMB being the substrate. Reaction was stopped using Sulphuric acid and OD read at 450 nm.

2.2.7.2 Indirect ELISA

Indirect ELISA is one of the best tools to study conformational antigen-antibodies interaction. Briefly, 96 well microtiter plates were coated with 100µl of amyloid beta peptide of concentration 5µg/mL with 100 mM Sodium Bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Discarding the content, plates were then coated with Super block (300µL), blocking solution and left overnight at 4°C. Plates were washed 5 times with PBS containing 0.05% of Tween and stored in the fridge.

2.2.7.3 Isolation of Aβ-specific auto-antibodies from IVIGs

NAbs-Aβ were isolated from IVIG using affinity chromatography (Balakrishnan et al., 2010). An affinity purification column was packed by SulfoLink gel (UltraLink, Pierce), couple with synthetic N-terminal cysteine-added Aβ (0.6 mg/ml) and resuspended in coupling buffer (50 mM Tris, 5 mM EDTA, pH 8.5). Blocking with cysteine (50 mM; resuspended in coupling buffer) and washing (1 M NaCl) preceded IVIG sample application. NAbs-Aβ were recovered with elution buffer (100 mM glycine, pH 2.5), immediately neutralized and used for further experiments.

2.2.8 Protein analysis

2.2.8.1 Alanine scanning mutagenesis

To characterise the epitope further, widely accepted alanine scan mutagenesis was used, where the natural amino acid sequence was replaced with Alanine (CH_3 side chain) as a site-specific point mutation. The rationale behind Alanine-scanning mutagenesis is that all interactions of a side chain except for C_β are eliminated (Tsiang et al., 1995).

2.2.8.2 Protein quantification using Nanodrop

Protein concentration was measured by A280 nm on a Nanodrop UV/visible spectrophotometer (Nanodrop®, US), using the respective buffer as blank and entering the Molar extinction coefficient and molecular weight of the protein/peptide.

2.2.8.3 Protein quantification using BCA-kit

Total protein concentration of brain extracts were quantified using BCA kit according to manufacturer's instructions (Pierce).

2.2.9 Cell culture

2.2.9.1 Maintenance of cell lines

N2A and BV2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) and F12 medium, respectively, supplemented with heat-inactivated 10% FBS, 100 U/ml Penicillin, and 100 µg/ml Streptomycin. Cells were grown at 37°C, 5% CO_2 aeration and 95% humidity. Cell culture work was performed under a sterile laminar flow safety cabinet using pre-sterilized materials and solutions. Cell splitting and passaging was performed twice a week obtaining confluency. Cells were washed with sterile PBS to remove cell debris and residues of FBS that might inhibit trypsin activity and incubated with 2 ml trypsin EDTA solution (0.5 mg/ml trypsin and 0.22 mg/ml EDTA) for 5 minutes at 37°C. Trypsin cleaves extracellular adhesion proteins and EDTA binds Ca^{2+} ions required for cell-cell adhesions formed by cadherins and thereby supports cell detachment. Cells were resuspended in 10 ml medium containing FBS and centrifuged at 500Xg for 5 mins. The pellet was resuspended in the respective medium and cells were seeded as single cell suspension in a dilution of 1:10. The medium was changed every two days to provide fresh nutrients, to remove toxic products and to prevent pH changes, which are indicated also by the pH indicator dye (phenol red) included in the medium.

2.2.9.2 Cell toxicity assay MTT assay (Aβ toxicity)

Cell proliferation was assessed by monitering the conversion of MTT to formazan. Mitochondrial dehydrogenase enzymes catalyze the reduction of MTT, which is therefore a measure of cell viability (Mosmann, 1983). For Aβ induced toxicity assay, cells were seeded at seeding densities of approximately $1x10^4$ cells/ml into 24 well

plates and allowed to adhere for 24 h. Prepared Aβ monomers, oligomers or mutant oligomers were mixed in a serum-free medium before treating the cells (Kayed et al., 2003). After 48 hours of treatment, cell viability was assessed by adding 500 µl of MTT (5mg/ml in PBS) and subsequently incubated for 4 hours. Medium was removed and cells were dissolved in sterile DMSA (500 µl) by incubated at 37°C for 30 min. The resulting absorbance at 550 nm was measured with a plate reader. Only the inner rows of the plate were used for the studies considering the medium evaporation rates at the periphery. The growth curve was constructed by plotting absorbance (blanked with DMSO) against time.

3 RESULTS

3.1 Epitope characterization

3.1.1 Comparison of commercial IVIG preparations for Aβ specific antibodies

Intravenous immunoglobulins (IVIG) are FDA-approved therapeutic agents for inflammatory and autoimmune disorders. Seven commercially available IVIGs (Table 2) were compared with respect to their differences in Aβ binding efficiency, using indirect ELISA. Two different lengths of Aβ peptide were used in the study $Aβ_{1-40}$ and $Aβ_{1-42}$. Commercially available IVIG showed considerable differences in the binding toward both Aβ peptides ($Aβ_{1-40}$ (Figure 5) and $Aβ_{1-42}$ (Figure 6)).

Table 2 Different brands of commercial IVIG preparations used in the study

IVIG Product	IgG (mg/ml)	Manufacturer
Gammagard	50	Baxter, Germany
Gammunex	100	Bayer, Germany
Intratect	50	Biotest Pharma, Germany
Kiovig	50 and 100*	Baxter, Germany
Octagam	50	Octapharma, Germany
Pentaglobin	50	Biotest Pharma, Germany
Sandoglobulin	50	CSL Behring, Germany

* Kiovig used for ELISA using $Aβ_{1-42}$ were supplied as 100 mg/ml concentration.

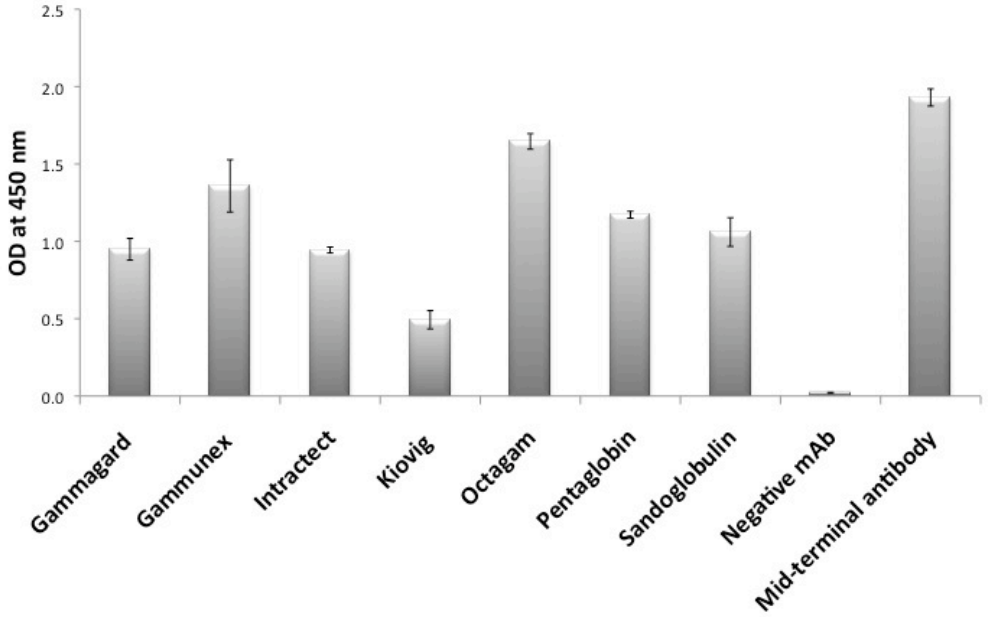

Figure 5 Comparison of binding affinities of commercial IVIG towards $A\beta_{1-40}$. Optical density represents binding of NAbs-Aβ towards $A\beta_{cys.1-40}$, measured using an ELISA coated with $A\beta_{cys.1-40}$. To account for the specificity of the technique, a monoclonal antibody specific for the mid-terminal region was added as a positive control and a non-specific monoclonal antibody was included as a negative control.

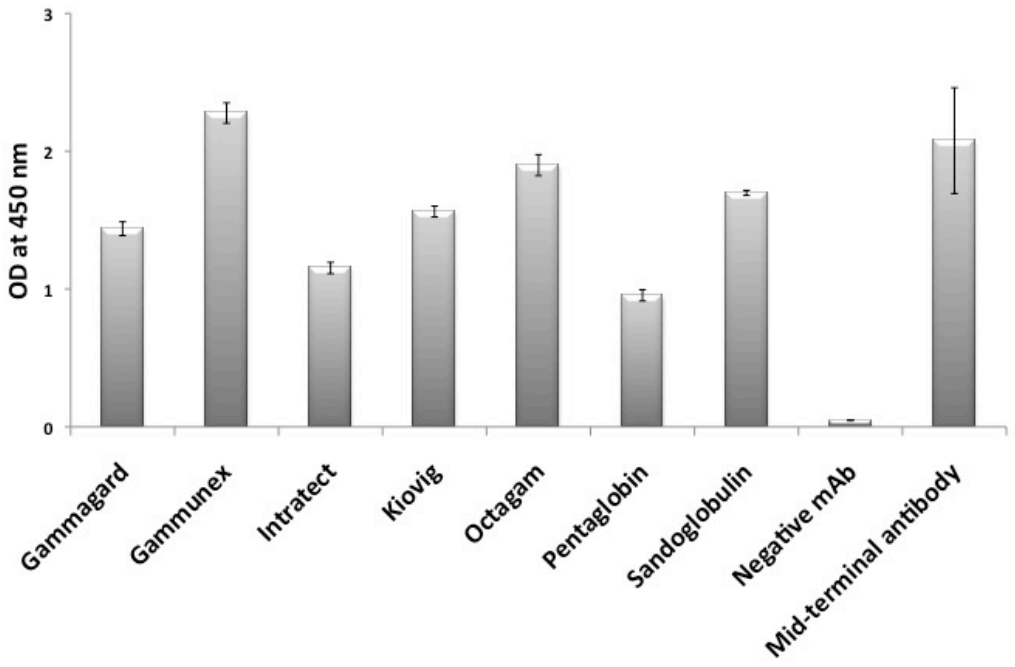

Figure 6 Comparison of binding affinities of commercial IVIG towards $A\beta_{1-42}$. OD represents the ELISA data demonstrating binding of NAbs-Aβ towards $A\beta_{1-42}$. Along with IVIG preparations, Aβ specific and non-specific monoclonal antibodies were used as positive and negative controls, respectively.

Among all IVIG products compared, Octagam and Gammunex showed the strongest binding to $A\beta_{1-40}$ and $A\beta_{1-42}$, reflecting the presence of more Aβ specific NAbs in their IgG pool. Sandoglobulin, Gammagard and Intratect showed similar binding efficiencies. Kiovig had the lowest concentration of NAbs-Aβ and hence the lowest binding affinity to $A\beta_{1-40}$, and moderate binding affinity towards $A\beta_{1-42}$. On the contrary, Pentaglobin showed the presence of more $A\beta_{1-40}$ specific NAbs than to $A\beta_{1-42}$ (Balakrishnan et al., 2010).

3.1.2 Determination of the Aβ domain-specificity of NAbs-Aβ

To identify the binding domain of Aβ that is recognized by the Aβ-affinity purified NAbs (NAbs-Aβ), the full-length sequence of $A\beta_{1-40}$ was compared with shorter truncated versions of the peptide using indirect ELISA. The peptide sequences used for the study comprised of: the full-length $A\beta_{1-40}$, reverse sequence of $A\beta_{40-1}$, truncated N-terminal ($A\beta_{1-15}$), mid-terminal ($A\beta_{10-28}$), and two C-terminal sequences ($A\beta_{20-38}$ & $A\beta_{25-40}$) of Aβ (Table 3). The full length and reverse sequences were used as positive and negative controls respectively. Other sequences had a prerequisite of 15 amino acids length and were chosen to represent different domains of Aβ.

$A\beta_{20-38}$ was included in the study following a previous report where $A\beta_{21-37}$ was shown to be the functional epitope for NAbs-Aβ (Przybylski M, 2008). NAbs-Aβ recognized $A\beta_{25-40}$ better than the rest of the domains of Aβ (Balakrishnan et al., 2010) (Figure 7). Interestingly, $A\beta_{20-38}$ that includes the same sequence as in $A\beta_{25-40}$ showed less binding.

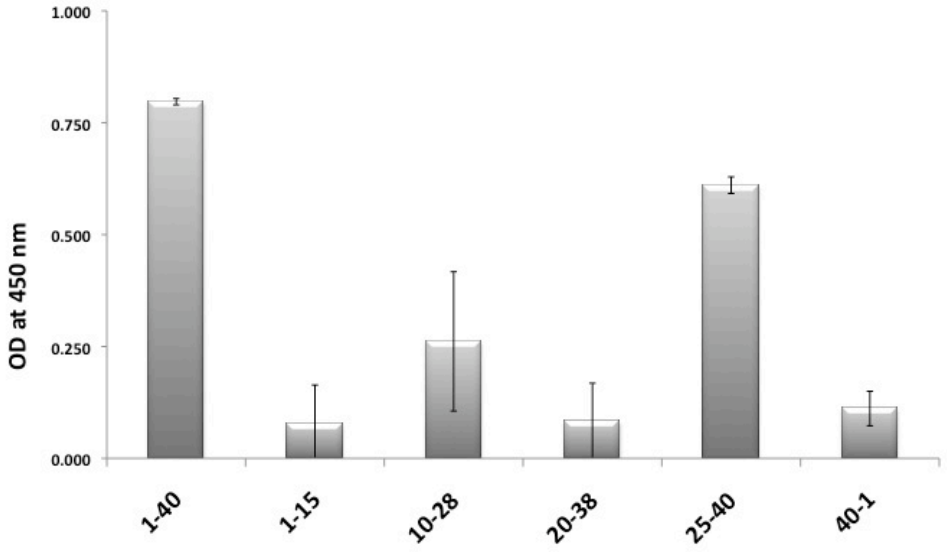

Figure 7 NAbs-Aβ recognize C-terminal Aβ fragment. Indirect ELISA, peptides were coated on plates and affinity NAbs (1mg/mL) were diluted to 1:12500 with duplicates. Bars represent the optical density measured at 450nm after the treatment with HRP labelled goat-anti-human secondary antibodies followed by addition of TMB substrate. Figure represents the one of the dilutions among three dilutions with consistent pattern and experiment was repeated thrice.

Table 3 Sequences of Aβ used in the experiment

Aβ 1-40 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
Aβ 1-15 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
Aβ 10-28 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
Aβ 25-40 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
Aβ 40-1 VVGGVMLGIIAGKNSGVDEAFFVLKQHHVEYGSDHRFEAD

Representation of human Aβ amino acid sequence in single letter code. The Aβ sequences used in the experiments are shown in white colour. The amino acids that are present in the original Aβ sequence

and excluded in the truncated Aβ sequences that are not used in the experiments are intentionally dimmed using blue letters.

3.1.3 Alanine scanning mutagenesis at the position 25 to 27 of Aβ C-terminal end decline their role as epitope

Alanine scanned $A\beta_{1-40}$ peptides, $A\beta_{1-40;\ G25A}$, $A\beta_{1-40;\ S26A}$ and $A\beta_{1-40;\ N27A}$, were analyzed used indirect ELISA. Additionally, truncated $A\beta_{23-31}$ and $A\beta_{1-40;\ S26S}$, where naturally occurring amino acid form *Levo* was substituted with *Dextro* isomers, were included in the studies (Figure 8).

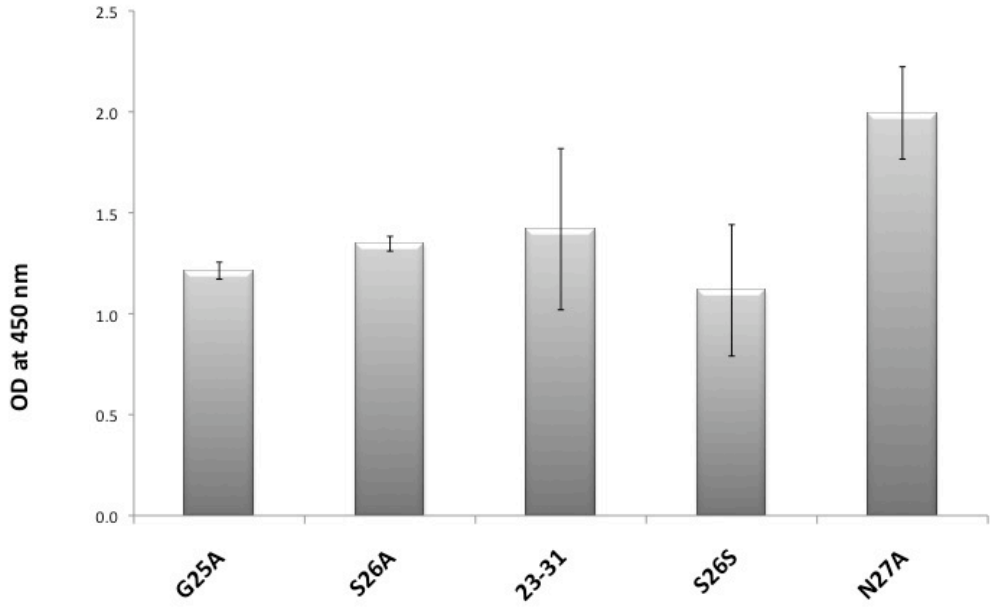

Figure 8 Alanine scanning mutagenesis at beginning of the C-terminal Aβ shows no significant decline in NAbs binding. Indirect ELISA, peptides were coated on plates and affinity NAbs (1mg/mL) were diluted (1XPBS) to 1:1000 with duplicates. Bars represent the optical density measured at 450nm. Figure represents the one of the dilutions among three dilutions with consistent pattern in three independent experiments.

Results showed that the Alanine substitution at positions 25, 26 or 27 did not affect the binding of NAbs. Moderately increased binding was found with $A\beta_{1-40;\ N27A}$ where replacing polar amino acid Asparagine was exchanged to non-polar Alanine. On the other hand, neither the replacement of *L*-Serine to *D*-Serine nor the Serine to Alanine affected the binding of NAbs-Aβ towards them. Interestingly, shorter $A\beta_{23-31}$ fragment showed relatively low binding compared to N27A mutant. Results suggested that the

NAbs-Aβ epitope region on Aβ was not between Aβ 1-27 as shown in the schematic diagram

Figure 9 Schematic diagram of Aβ sequence recognized by NAbs-Aβ. Figure displays the full-length Aβ amino acid one letter sequence and the expected epitope region in black colour with green glow. Alanine scanned positions tested in the experiment are given in yellow colour, where red coloured glow indicates that those positions are not representing the epitope. Alanine scans at positions 25, 26 and 27 suggests that these three amino acids are not representing the epitope region on Aβ detected by NAbs-Aβ. Aβ sequences that are not used in the experiments are intentionally dimmed using blue letters.

3.1.4 NAbs-Aβ specifically recognizes the C-terminal end of Aβ 28-42

The far C-terminal end of Aβ sequence was analyzed to further explore the epitope region. Truncated $Aβ_{42}$ was preferred to truncated $Aβ_{40}$ to avoid the extreme shortening of the peptide's length in the studies. The binding of NAbs-Aβ with far C-terminal end comparing 3 shorter peptide sequences $Aβ_{26-42}$, $Aβ_{28-42}$ and $Aβ_{30-42}$ was determined using indirect ELISA. Synthetic $Aβ_{30-42}$ had solubility issues while resuspending in the well coating buffer; this may be due to the presence of highly hydrophobic nature of C-terminal sequence of the Aβ peptide.

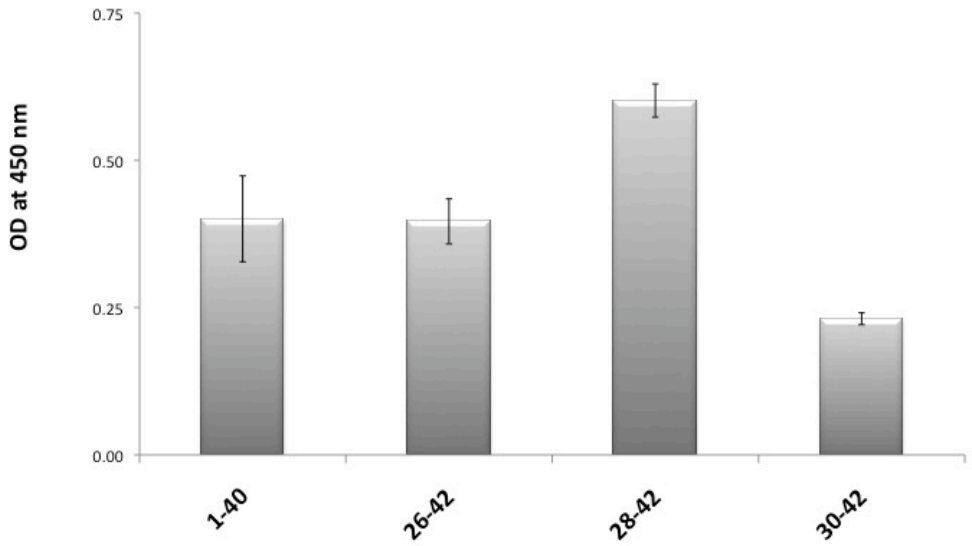

Figure 10 Comparison of far C-terminal Aβ shows increased binding of NAbs (1: 250 dilution with duplicates). Figure represents ELISA data from one of the dilutions among three dilutions with consistent pattern in three independent experiments.

Data (Figure 10) demonstrates that the NAbs-Aβ recognize $A\beta_{28-42}$ better than $A\beta_{1-40}$, $A\beta_{26-42}$ and $A\beta_{30-42}$, which is in line with the previous Alanine-scanning mutagenesis results (Figure 8).

3.1.5 Conformation specificity of Aβ-specific NAbs

To extend the understanding of the NAbs-Aβ binding on the Aβ, alanine-scanned C-terminal sequences were compared using indirect ELISA. To avoid the solubility issues observed with $A\beta_{30-42}$ and to maintain the prerequisite of at least 14 amino acids length, the alanine scans were done at positions 30, 33 and 35 of $A\beta_{28-42}$, respectively. Despite the fact that alanine at position 30 could not represent the epitope region, glycine was substituted to find out whether the improved solubility helps NAbs-Aβ binding. Accordingly, alanine at position 30 was exchanged to glycine. Alanine scan at 35th position was done to determine whether methionine represent the Aβ epitope region for NAbs-Aβ. Additionally, to check the influences of the coating buffer, two different coating buffers were used. For this study, PBS (pH 7.4) that helps Aβ aggregation and 0.1 M Sodium carbonate buffer (pH 9.6), a pH range that restrict Aβ aggregation were used (Balakrishnan et al., 2010). The full-length and truncated forms of Aβ were coated with two different well coating buffers with different pH. Alanine scanned peptides were coated with 0.1 M Sodium carbonate.

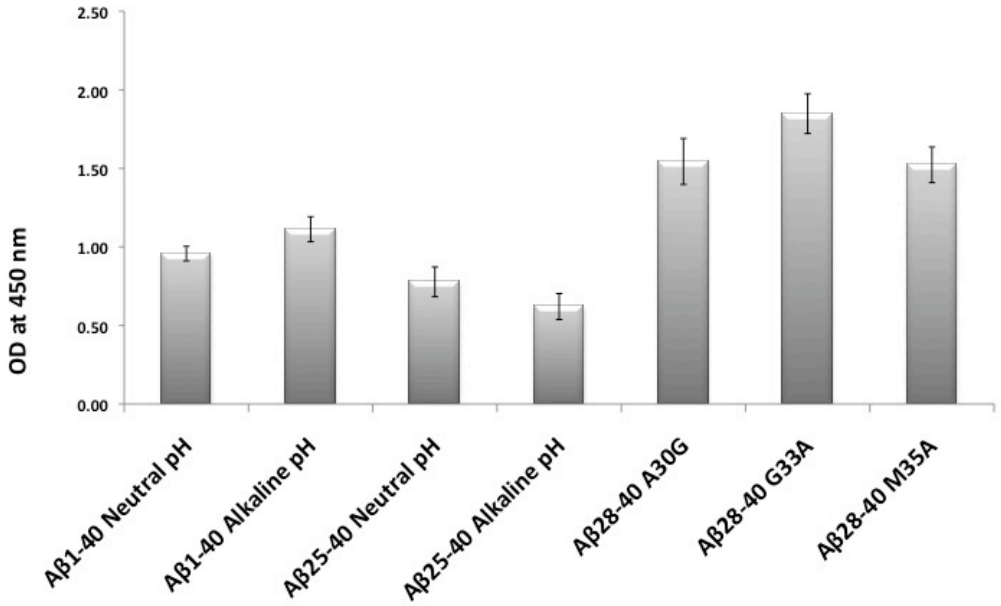

Figure 11 Comparison of Alanine-scanned C-terminal Aβ and effect of the pH of well coating buffer. Indirect ELISA, for $A\beta_{1-40}$ and $A\beta_{25-40}$ peptides we used PBS (pH 7.4) and 0.1M Sodium

carbonate buffer (pH 9.6) as well coating buffer. The alanine-scanned peptides were coated with 0.1M Sodium Carbonate buffer and affinity NAbs (1mg/mL) were diluted with 1XPBS to 1: 250 with duplicates. Bars represent the optical density measured at 450 nm after the treatment with HRP labelled goat-anti-human secondary antibodies followed by addition of TMB substrate. Figure represents the one of the dilutions among three dilutions with consistent pattern in three independent experiments.

The ELISA data shows that the different coating buffers did not influence the binding of NAbs-Aβ towards the Aβ. Interestingly, the substitution of Alanine at position 30 of the Aβ peptide increased the binding of affinity purified NAbs. Increased binding of the antibodies towards the truncated peptide may be due to its effective display of the epitope. But according to the (Figure 11), when compared to the other truncated alanine-scanned peptides Alanine replacement at the position 30 increases the binding of the NAbs-Aβ.

3.1.6 Influence of cysteine in NAbs-Aβ binding

Previous experiment suggested the possibility of either the Aβ oligomerization or the conformational change due to hydrophobic amino acid inclusion might influence the NAbs-Aβ binding. Further, experiment was designed to investigate the effect of oligomerization and the possible conformational changes due to non-natural amino acid inclusion. Cysteine was included at the N-terminal sequence of Aβ. Cysteine was chosen to induce oligomerization because of its tendency to form cystine disulphide bonds (O'Nuallain et al., 2010). Another rationale behind the choice of cysteine was its usage in the Aβ affinity purification columns (Balakrishnan et al., 2010) see section (2.2.7.3). Influence of the cysteine was studied by comparing WT Aβ to N-terminal cysteine added sequences. For this study, NAbs-Aβ binding efficiencies towards WT and N-terminal sequences of full length ($Aβ_{1-40}$ and N-cys-$Aβ_{1-40}$), ($Aβ_{1-15}$ and N-cys-$Aβ_{1-12}$), mid-terminal ($Aβ_{10-28}$ and N-cys-$Aβ_{10-38}$), and C-terminal Aβ sequences ($Aβ_{20-38}$, $Aβ_{25-40}$, $Aβ_{28-42}$, and N-cys-$Aβ_{25-40}$), were compared.

In addition, $Aβ_{20-38;\ V39C}$ and $Aβ_{28-42;I31A,L34A,\ V40A}$ were included in the studies. Previously $Aβ_{20-38}$ showed less binding towards NAbs-Aβ compared to $Aβ_{25-40}$, which suggested valine at position 39 or 40 might either contribute as epitope or assist oligomerization (Figure 10). Addition of cysteine was expected to improve the aggregation by forming inter-molecular disulphide bonds between cysteine residues and thus help oligomerization. Cysteine was added at 39th position of $Aβ_{20-38;}$ ($Aβ_{20-38;V39C}$) to explore whether the binding of NAbs-Aβ could be restored. Alanine

scanning was done on three hotspots ($A\beta_{28\text{-}42;I31A,L34A}$ sequence) to find whether any one of the three amino acids represents the epitope region for NAbs-Aβ. Reverse sequence of Aβ (i.e. $A\beta_{40\text{-}1}$) was included as negative control.

The rationale behind Alanine-scanning mutagenesis at 31, 34 and 40th positions of Aβ peptide is that, NAbs-Aβ recognized sequence between 30-40 in our previous experiments (Figure 11). Alanine substitution at positions 33 and 35 did not influence the binding of the NAbs-Aβ. Glycine, with H as its functional group, cannot act as an epitope and is present at 30th, 33rd, 37th and 38 positions. Replacement of Methionine at position 35 to Alanine did not influence NAbs-Aβ binding (Figure 11). As per Petkova et al's observation, Isoleucine at the position 32, Valine at positions 36 and 39 are involved in aggregation process and we assumed these amino acids would not be free to represent an epitope.

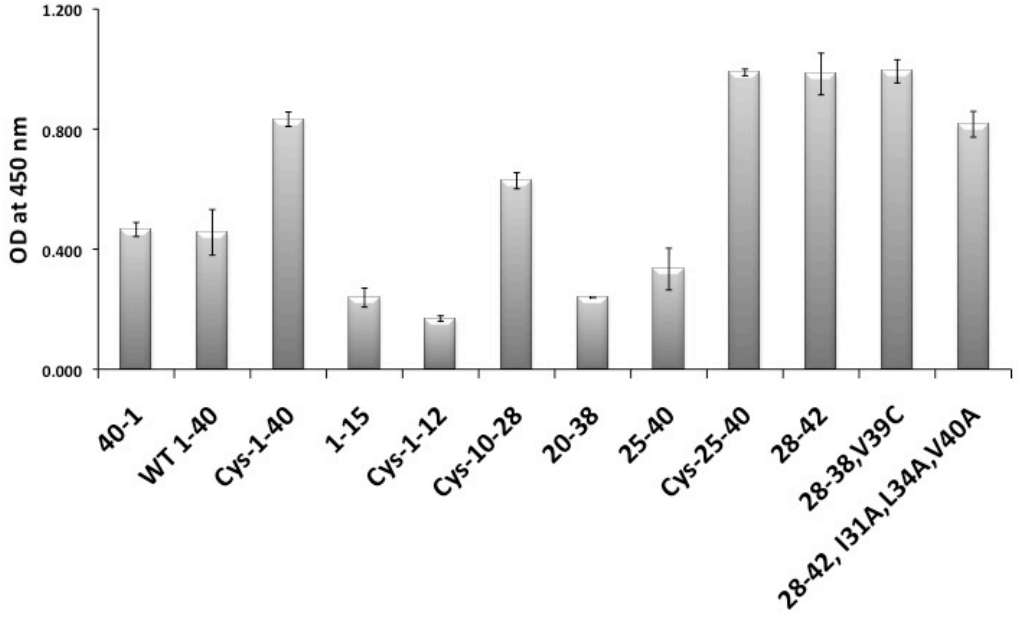

Figure 12 Comparison of truncated peptides with and without cysteine and suspected epitope region. Indirect ELISA, peptides were coated on 96 well plate with 0.1M Sodium carbonate buffer (pH 9.6) as well coating buffer. The affinity NAbs-Aβ (1mg/mL) were diluted with 1XPBS to 1: 4000 with duplicates. Bars represent the optical density measured at 450 nm after the treatment with HRP labelled goat-anti-human secondary antibodies followed by addition of TMB substrate. Figure represents the one of the dilutions among three dilutions with consistent pattern in three independent experiments.

The ELISA data demonstrates the influence of the additional cysteine residue at the N-terminal end of Aβ for the recognition of NAbs-Aβ (Figure 12). N-terminally cysteine added C-terminal Aβ peptides were recognized better by the NAbs-Aβ compared to the N-terminal sequence with or without the cysteine. This suggested that both the C-

terminal sequence and the Cysteine are important for the NAbs binding. In turn, N-terminal cysteine helps the peptide to oligomerize, suggesting NAbs-Aβ recognize oligomerized forms of the Aβ peptide. In addition, $Aβ_{28-38}$ was recognized by the NAbs-Aβ with a cysteine replacement at the 39th position. This confirms our assumption that the Valine at 39th or 40th positions helps in oligomerization and does not act as epitope. This could also be the reason why $Aβ_{20-38}$ was not recognized by the NAbs-Aβ. Alanine scanning mutagenesis at the positions 31, 34 and 40th position did not affect the NAbs binding suggesting those three positions are not representing the epitope region.

Figure 13 Schematic diagram of Aβ sequence recognized by NAbs-Aβ. Figure displays the full-length Aβ amino acid one letter sequence and the expected epitope region in black colour with green glow. Alanine scanned positions tested in the experiment are given in yellow colour, where red coloured glow indicates that those positions are not representing the epitope. Alanine scans at positions 31, 34 and 40 suggests that these three amino acids are not representing the epitope region on Aβ detected by NAbs-Aβ. Aβ sequences that are not used in the experiments are intentionally dimmed.

In summary, the results obtained in these experiments left three possible amino acids positions as potential epitope region (Figure 13).

3.1.7 Isoleucine at position 32 plays a crucial role as epitope region for NAbs-Aβ binding

To further understand the NAbs-Aβ binding towards Aβ addtion of cysteine, alanine scan mutagenesis and amino acid substition analysis were performed. Full length Aβ with additional cysteine at N-, C- and both N & C-terminal were analysed to study the influence addition of cysteine at the C-terminal as a continuation of the previous experiment .

Truncated $Aβ_{28-42}$ with three alanine substiutions at positions 32, 35 and 40 was included in the study along with WT $Aβ_{1-40}$ and $Aβ_{40-25}$ as positive and negative controls respectively. The rationale behind the triple mutant $Aβ_{28-42;\ I32A,\ M35A,V40A}$ are: previous results suggested that the epitope region should be between 30-40 (Figure 12), Glycine at positions 30, 33 and 37 cannot act as an epitope, positions 31 and 34 are not epitope according to the alanine-scanning . Valine at 40th position was included

in the studies, considering its role in oligomerization, whose exclusion might affect the NAbs-Aβ binding. As another approach, a scrambled sequence of $Aβ_{28-42}$ was used, where a jumbled sequence except the epitope region could yield NAbs-Aβ binding. In this scrambled sequence, all the amino acids except the 31, 35 and 40th position amino acids were substituted with other amino acid with equal polarity and hydrophobicity.

Further, glycine at positions 29 and 33 of full length $Aβ_{1-40}$ was exchanged to isoleucine. Replacement was based on previous observation where binding substitution of hydrophobic amino acid alanine instead of glycine improved efficiency of NAbs-Aβ (Figure 11). Isoleucine was substituted to improve the hydrophobicity of the C-terminal end and thereby helping Aβ oligomerization. Previously, intramolecular salt bridge formation between aspartate and lysine was reported (Petkova et al., 2002). In this regard, full length $Aβ_{1-40}$ with an exchange of positions between aspartate at 23rd position and lysine at 28th position were used. The rationale was to explore whether the distrubance of the salt bridge formation affect the NAbs-Aβ binding. Additionally, lysine at 28th position was exchanged to aspartate on the full length $Aβ_{1-40}$ sequence along with alanine-scans at positions 32 and 40. This triple mutation was used in the experiment to study whether inclusion of aspartic acid improves the NAbs-Aβ binding.

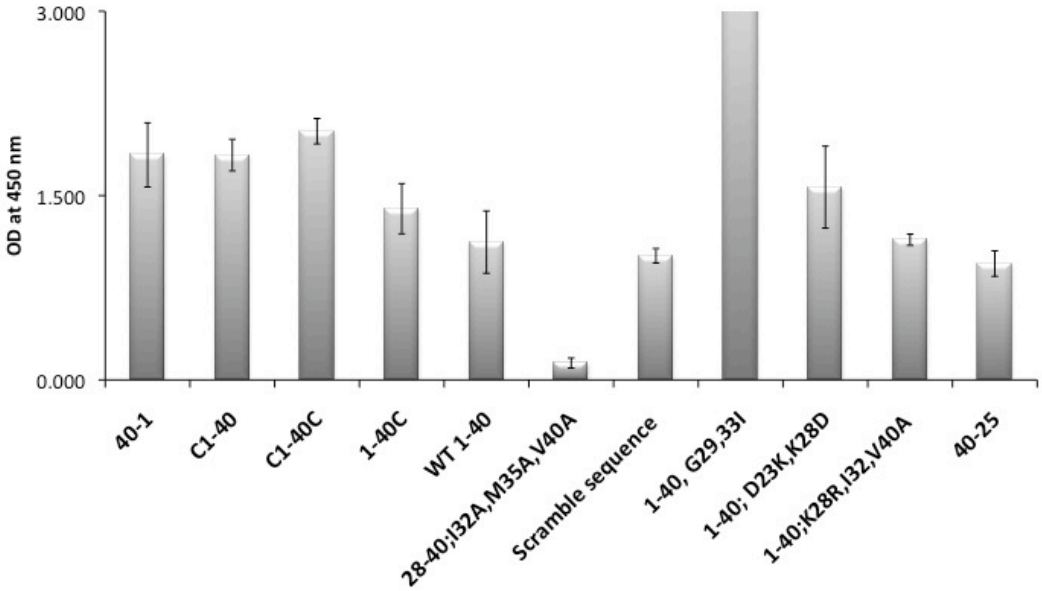

Figure 14 Comparison of Alanine-scanned C-terminal Aβ and cysteine addition at N & C-terminals. Indirect ELISA, cysteine added, alanine-scanned, and scramble sequences peptides were coated with 0.1M Sodium Carbonate buffer. Affinity NAbs-Aβ (1mg/mL) were diluted with 1XPBS to

1: 250 with duplicates. Bars represent the optical density measured at 450 nm after the treatment with HRP labelled goat-anti-human secondary antibodies followed by addition of TMB substrate. Figure represents the one of the dilutions among three dilutions with consistent pattern in three independent experiments.

Results clearly showed that the addition of cysteine to Aβ improved NAbs-Aβ binding compared to the WT Aβ (Figure 14). Despite the addition of cysteine improves the NAbs-Aβ binding, C-terminal cysteine added sequence showed less binding efficiency toward NAbs-Aβ. The triple alanine scanned sequence $A\beta_{28\text{-}42;\ I32A,\ M35A,V40A}$ showed remarkably less binding towards NAbs-Aβ. On the contrary, the binding efficiency of the NAbs-Aβ towards the scramble sequence was not completely abolished. $A\beta_{1\text{-}40;I32A,M35A,V40A}$. Interestingly, NAbs-Aβ showed comparatively high binding efficiency towards isoleucine substituted $A\beta_{1\text{-}40;G29,33I}$. Both $A\beta_{1\text{-}40;D23K,K28D}$ and $A\beta_{1\text{-}40;D23R,I32A,V40}$ showed similar binding to NAbs-Aβ as WT $A\beta_{1\text{-}40}$. The reverse sequence $A\beta_{40\text{-}25}$ showed less binding efficiency towards NAbs-Aβ.

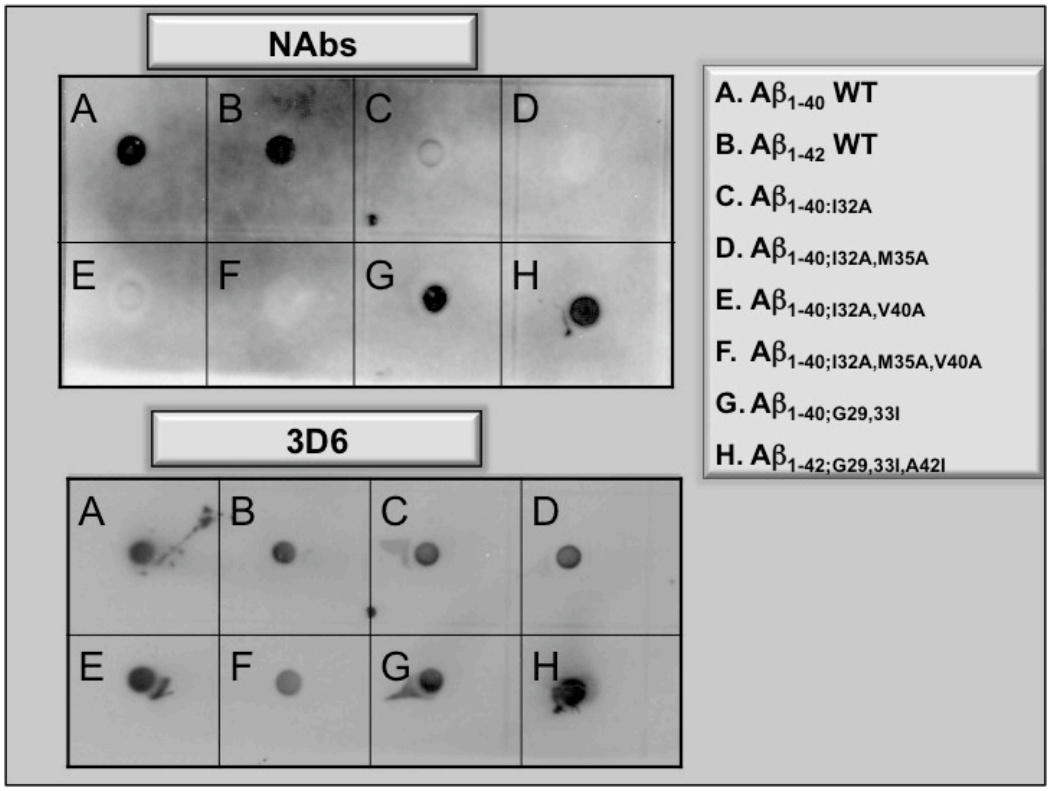

Figure 15 Dot blot showing Alanine scanning at position 32 replacing Isoleucine completely abolishes NAbs binding. The stripped blot was then treated with N-terminal specific 3D6 antibodies that recognize all the scanned peptides. Synthetic wild type and alanine scanned peptides (2μg) were dotted on Nitrocellulose membrane followed NAbs-Aβ treatment for O/N at 4°C, the same blot was treated with stripping buffer and incubated for 1 hour with 3D6.

To confirm the ELISA data where alanine substitution at 32nd position of Aβ decreased NAbs-Aβ binding, Dot blot analysis was done. For this study, full-length Aβ with alanine scans were done primarily at position 32. To find out whether the decreased binding was due to a single amino acid at a specific position or a collection of all three amino acids alanine scans were planned accordingly. Peptides $Aβ_{1-40}$, $Aβ_{1-42}$, $Aβ_{1-40;I32A}$, $Aβ_{1-40;I32A,M35A}$, $Aβ_{1-40;I32A,M35A,V40A}$, $Aβ_{1-40;G29,33I}$, and $Aβ_{1-42;G29,33I,A42I}$ were analyzed using Dot blots (Figure 15).

Results confirm that isoleucine at position 32 represent the epitope for NAbs-Aβ. Interestingly, all peptide sequences with alanine scan at 32nd position completely abolished NAbs-Aβ binding. $Aβ_{1-40;G29,33I}$, and $Aβ_{1-42;G29,33I,A42I}$ and the positive controls $Aβ_{1-40}$, and $Aβ_{1-42}$ showed the expected signal. Removal of antibodies from the membrane and re-probing using N-terminal specific 3D6 antibodies showed the peptides were spotted on the membrane. In summary, epitope mapping clearly demonstrated the role of Lys28 and Ile32 by comparing a list of full length (Figure 16) and truncated peptides (Figure 17). Especially, Ile32 plays a very vital role as an amino acid representing Aβ epitope region for NAbs-Aβ.

Peptide sequence	Label	Signal
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	WT $Aβ_{-1-40}$	+
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	WT $Aβ_{1-42}$	++
CDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	$Aβ_{C-1-40}$	++
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVC	$Aβ_{1-40-C}$	++
CDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVC	$Aβ_{C-1-40-C}$	++
DAEFRHDSGYEVHHQKLVFFAEDVASNKGAIIGLMVGGVV	$Aβ_{1-40;G25A}$	++
DAEFRHDSGYEVHHQKLVFFAEDVGANKGAIIGLMVGGVV	$Aβ_{1-40;S26A}$	++
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	$Aβ_{1-40;S26S*dextro}$	++
DAEFRHDSGYEVHHQKLVFFAEDVGSNKIAIILMVGGVV	$Aβ_{1-40;G29,33I}$	+++
DAEFRHDSGYEVHHQKLVFFAEDVGSNKIAIILMVGGVVIA	$Aβ_{1-42;G29,33I}$	+++
DAEFRHDSGYEVHHQKLVFFAEDVGSNKIAIILMVGGVVII	$Aβ_{1-42;G29,33I,A42I}$	+++
DAEFRHDSGYEVHHQKLVFFAEKVGSNDGAIIGLMVGGVV	$Aβ_{1-40;D23K,K28D}$	++
DAEFRHDSGYEVHHQKLVFFAEDVGSNRGAIAGLMVGGVA	$Aβ_{1-40;K28R,I32A,V40A}$	++
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAAIGLAVGGVA	$Aβ_{1-40;I32A,M35A,V40A}$	-
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAAIGLMVGGVA	$Aβ_{1-40;I32A,V40A}$	-
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAAIGLAVGGVV	$Aβ_{1-40;I32A,M35A}$	-
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAAIGLMVGGVV	$Aβ_{1-40;I32A}$	-

Figure 16 List of Full length peptides used and their binding signal to NAbs-Aβ. Truncated sequence with substitution mutations and signal intensity given by indirect ELISA using NAbs.

Peptide sequence	Label	Signal
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	WT $A\beta_{-1-40}$	+
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	WT $A\beta_{1-42}$	++
DAEFRHDSGYEV	$A\beta_{1-12}$	+
DAEFRHDSGYEVHHQ	$A\beta_{1-15}$	+
YEVHHQKLVFFAEDVGSNK	$A\beta_{10-28}$	+
FAEDVGSNKGAIIGLMVGG	$A\beta_{20-38}$	+
DVGSNKGAI	$A\beta_{20-31}$	++
GSNKGAIIGLMVGGVV	$A\beta_{25-40}$	+++
SNKGAIIGLMVGGVVIA	$A\beta_{26-42}$	++
KGAIIGLMVGGVVIA	$A\beta_{28-42}$	+++
AIIGLMVGGVVIA	$A\beta_{30-42}$	++
KGGIIGLMVGGVVIA	$A\beta_{28-42;A30G}$	+++
KGAIIALMVGGVVIA	$A\beta_{28-42;G33A}$	+++
KGAIIGLAVGGVVIA	$A\beta_{28-42;M35A}$	+++
KGAIIGLMVGGVVC	$A\beta_{28-38;V39C}$	++
KGAAIGAMVGGVAIA	$A\beta_{28-42:I31A,L34A,V40A}$	++
KGAAIGAMVGGVAIA	$A\beta_{28-40:I32A,M35A,V40A}$	-

Figure 17 Truncated sequence with substitution mutations and signal intensity given by indirect ELISA using NAbs-Aβ

3.2 Oligomerization of Amyloid beta

3.2.1 Inclusion of N-terminal cysteine helps Aβ dimerization

Data from previous experiments indicates addition of cysteine and there by increased Aβ, improves binding of NAbs-Aβ toward the Aβ. Further, experiments were designed to study the Aβ oligomerization driven by the addition of N-terminal cysteine to WT Aβ sequence. In this study, several parameters like, addition of cysteine, influence of pH of the solution, incubation time, storage parameters and physical force (like shaking or stirring) of the peptide solution were studied.

After oligomerization experiments, ELISA and SDS-PAGE were performed to determine the NAbs-Aβ binding and approximate oligomeric form of every particular sample, respectively (Figure 18).

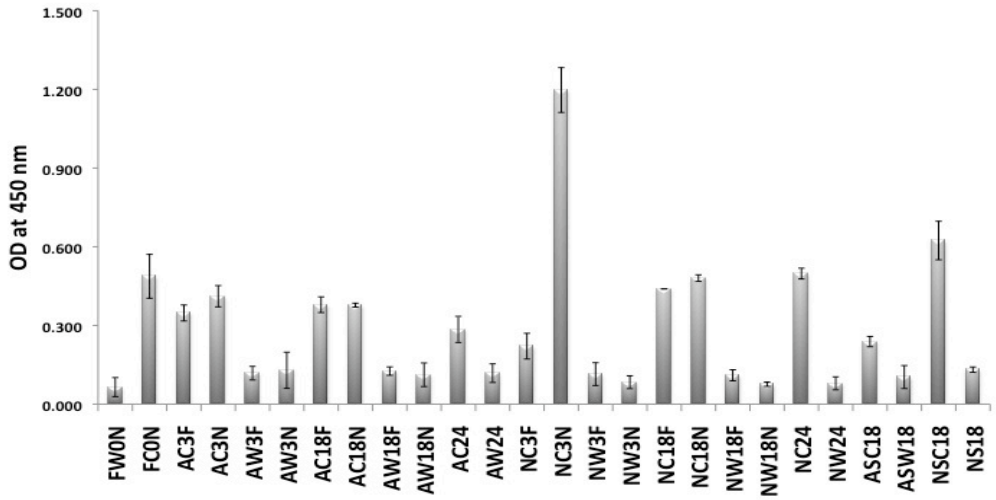

Figure 18 Time course experiment: Aβ peptide treated with different parameters shows the NAbs-Aβ recognize particularly N-Cys-Aβ$_{1-40}$.

Abbreviation	Expansion
FW0N	Fresh WT 1-40, 0 hours incubation.
FC0N	Fresh Cys-1-40, 0 hours incubation
AC3F	Acidic buffer, Cys-1-40, 3 hours & Frozen after incubation
AC3N	Acidic buffer, Cys-1-40, 3 hours & stopped with Ammonia
AW3F	Acidic buffer, WT 1-40, 3 hours & Frozen after incubation
AW3N	Acidic buffer, WT 1-40, 3 hours & stopped with Ammonia
AC18F	Acidic buffer, Cys 1-40, 18 hours & Frozen after incubation
AC18N	Acidic buffer, Cys 1-40, 18 hours & stopped with Ammonia
AC24	Acidic buffer, Cys-1-40, 24 hours incubation
AW24	Acidic buffer, WT 1-40, 24 hours incubation
NC3F	Neutral buffer, Cys-1-40, 3 hours & Frozen after incubation
NC3N	Neutral buffer, Cys-1-40, 3 hours & stopped with Ammonia
NW3F	Neutral buffer, WT 1-40, 3 hours & Frozen after incubation
NW3N	Neutral buffer, WT 1-40, 3 hours & stopped with Ammonia
NC18F	Neutral buffer, Cys 1-40, 18 hours & Frozen after incubation
NC18N	Neutral buffer, Cys 1-40, 18 hours & stopped with Ammonia
NC24	Neutral buffer, Cys-1-40, 24 hours incubation
NW24	Acidic buffer, WT 1-40, 24 hours incubation
ASC18	Acidic buffer, Cys-1-40, 18 hours incubation with stirring
ASW18	Acidic buffer, WT 1-40, 18 hours incubation with stirring
NSC18	Neutral buffer, Cys-1-40, 18 hours incubation with stirring
NSW18	Neutral buffer, WT 1-40, 18 hours incubation with stirring

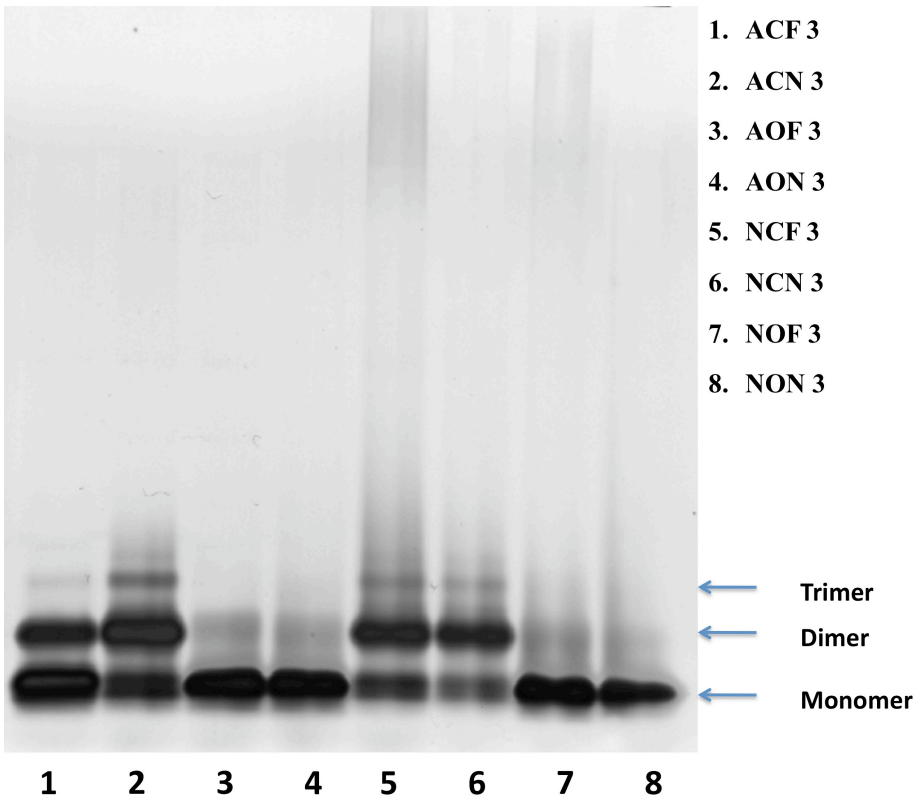

Figure 19 Influence of different parameters on $A\beta_{1-40}$ oligomerization after 3 hours incubation. Aβ with and without cysteine were incubated in acidic and neutral pH for 3 hours. Subsequently samples were electrophoresed and silver stained.

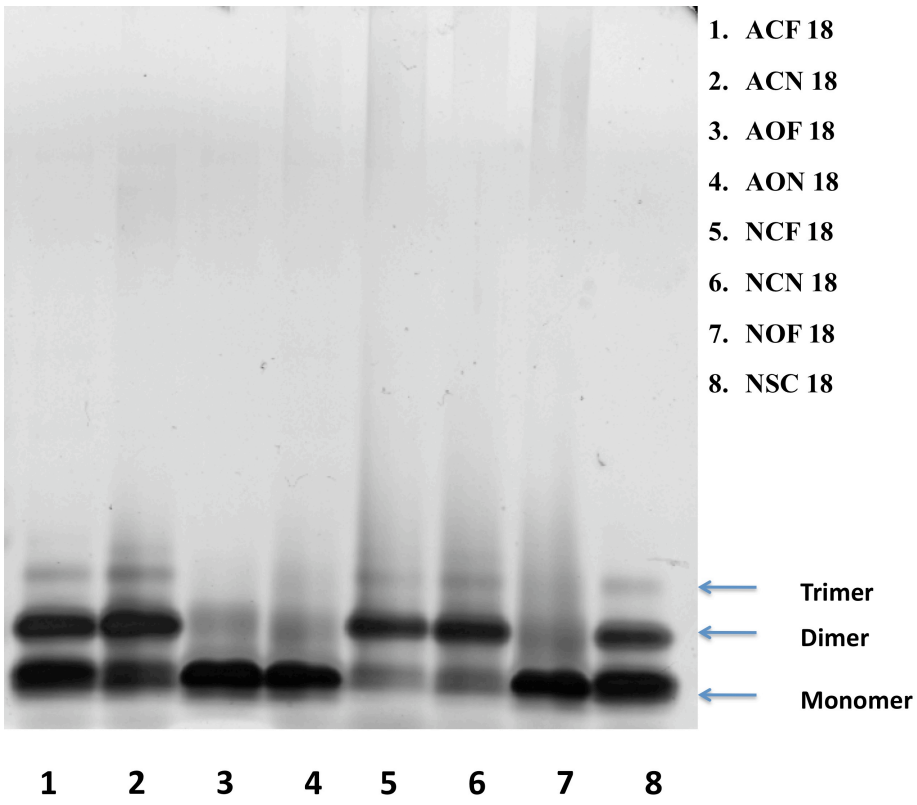

Figure 20 Influence of different parameters on $A\beta_{1-40}$ oligomerization after 18 hours incubation. Aβ with and without cysteine were incubated in acidic and neutral pH for 18 hours. Subsequently samples were electrophoresed and silver stained to detect the oligomerization.

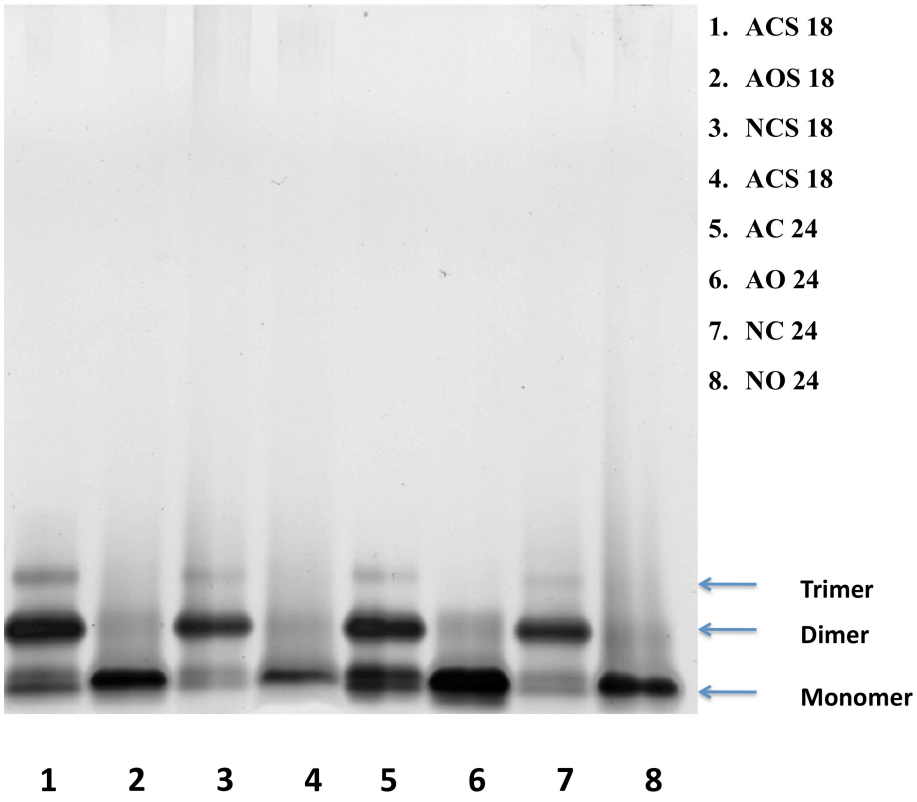

Figure 21 Influence of different parameters on $A\beta_{1-40}$ oligomerization after 18 and 24 hours incubation. Aβ with and without cysteine were incubated in acidic and neutral pH for 18 and 24 hours. Subsequently samples were electrophoresed and silver stained to detect the oligomerization

3.3 NAbs–Aβ recognizes Aβ conformational epitope

It was previously shown that NAbs-Aβ recognize C-terminus of Aβ peptide and binds to Aβ oligomers better than monomeric Aβ. Aβ undergo different conformations, in this regard, knowledge about the conformational epitope of NAbs-Aβ becomes crucial.To extend out the understanding of Aβ epitope, conformationally fixed Aβ peptides were analyzed. Click peptides or *O*-acyl isopeptide method were used to serve this purpose. Click peptides form amide bonds instead of peptide bonds and slow down the aggregation rate of the amyloid peptide (Sohma and Kiso, 2006). This property of click peptides thereby provide the conformational stability of Aβ and helps to study the antigen-antibody interaction.

In this study, NAbs-Aβ binding towards conformationally stable truncated $A\beta_{28\text{-}40}$ peptides, particularly depicting linear, parallel, anti-parallel and fibrill like epitopes, were analyzed. In total 24 Aβ click peptides were used, which were named as Mini-Aβ (MA) peptides. Indirect ELISA was used to compare the NAbs-Aβ binding toward MA peptides.

The conformationally stable truncated MA peptides described in this chapter were prepared by Dr. A. Roeder in the research group of, our collaborator, Prof. Dr. A. Geyer, Department of Peptide chemistry, Philipps-University of Marburg. Description of the preparation of the peptides are presented in the thesis of Dr. A. Roeder (Roeder, 2012). The ideas and the results developed has been submitted for a patent application (R. Dodel, 2011).

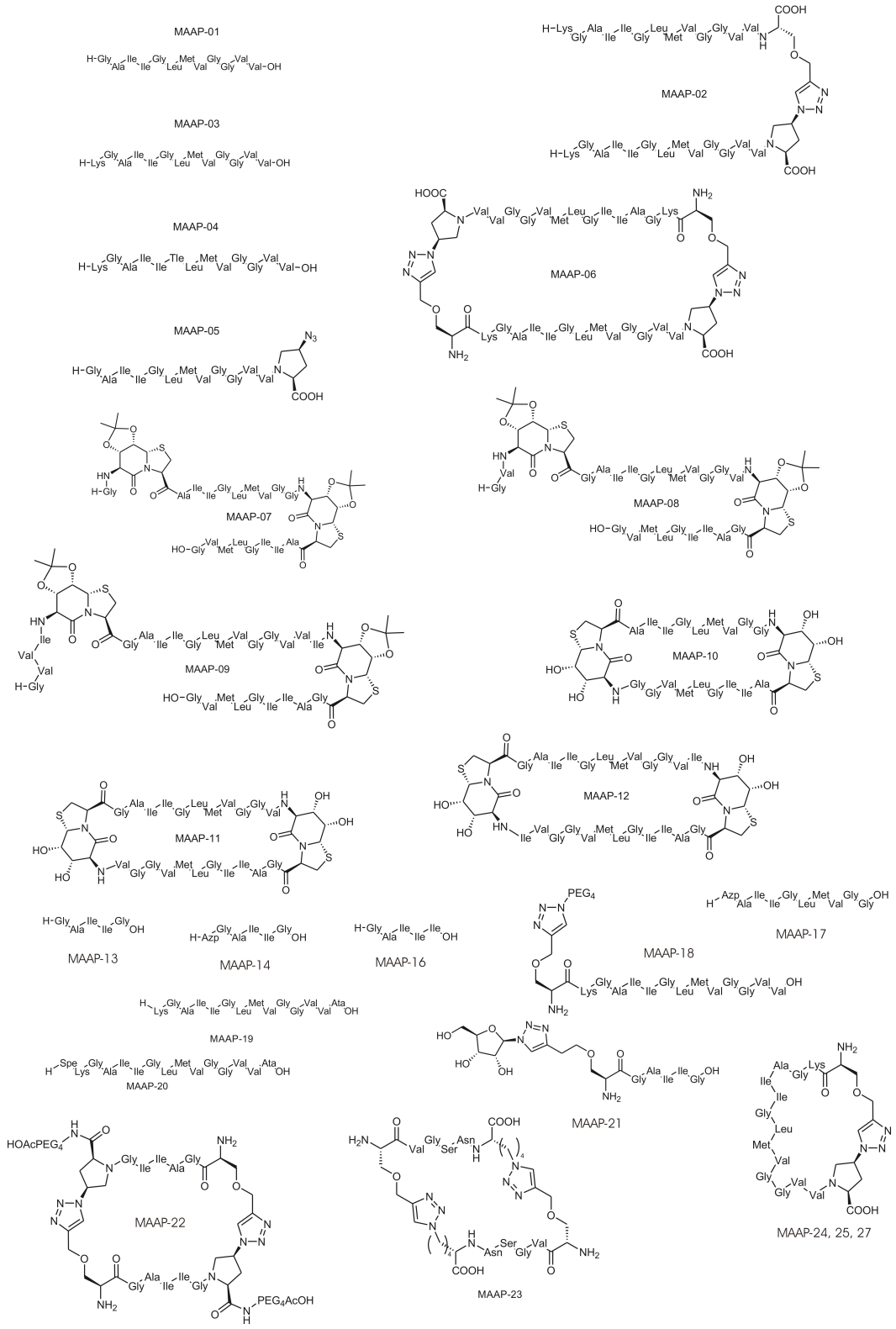

Figure 22 Chemistry of MA peptides. Short peptides were designed according to the epitope domain recognized by NAbs-Aβ. Synthetic peptides were produced using click peptides to fix the conformation of the peptide stable. MA 01, 03, 04, 05, and 13-21 represent epitope region as linear, and rest of the peptide display dimeric conformations, respectively.

3.3.1.1 NAbs-Aβ recognize dimeric conformation better than monomers

In order to identify the binding of NAbs-Aβ towards the first set of 12 MA peptides, MA 01 to MA 12 were compared using indirect ELISA. Considering the practical difficulties in synthesizing the click peptides for full length of Aβ, C-terminal sequences were chosen for the experiments. Additionally, the characterization (NAbs-Aβ binding) of shorter form of Aβ peptides were done intended to develop a candidate for active vaccination approach.

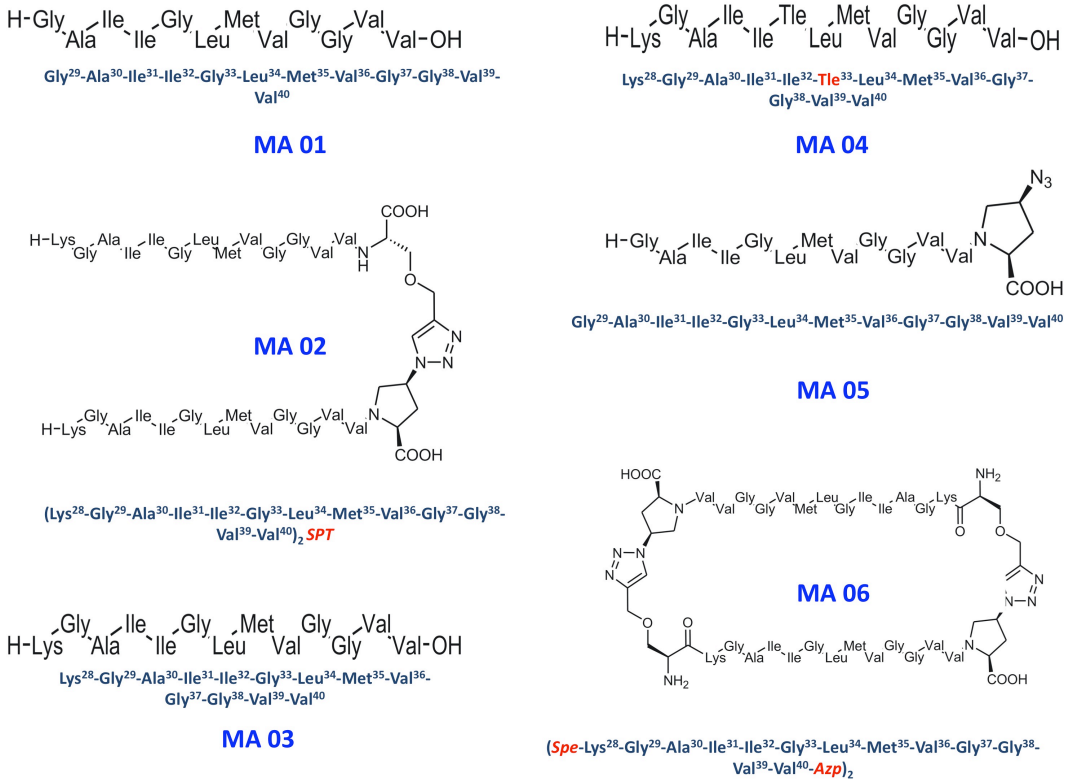

Figure 23 Chemistry of MA peptides 01-06

MA 01 represents a linear $A\beta_{29\text{-}40}$ peptide. MA 02 chemistry provides a flexible, dimeric, open-chain $A\beta_{28\text{-}40}$ linked at the C-terminal end using click chemistry L-Serine propargyl ether (Spe). MA 03 display monomeric linear $A\beta_{28\text{-}40}$ sequence. Previous epitope characterization showed improved binding of NAbs-Aβ due to the inclusion of an hydrophobic amino acid at 33rd position of Aβ (Figure 11). To further study whether the similar effect could be obtained using L-*tert*-leucine (t-leucine), which is more hydrophobic compared to leucine, was used as MA 04. MA 05 also represent $A\beta_{29\text{-}40}$ peptide with additional C-terminal proline derivative. MA 06 is a cyclic anti-parallel dimer with flexible linkers Spe and cis-4-Azido-L-proline (Azp).

The peptide strands can move against each other but remains in an anti-parallel alignment. The chemistry of MA 01-06 are given in the figure above (Figure 23).

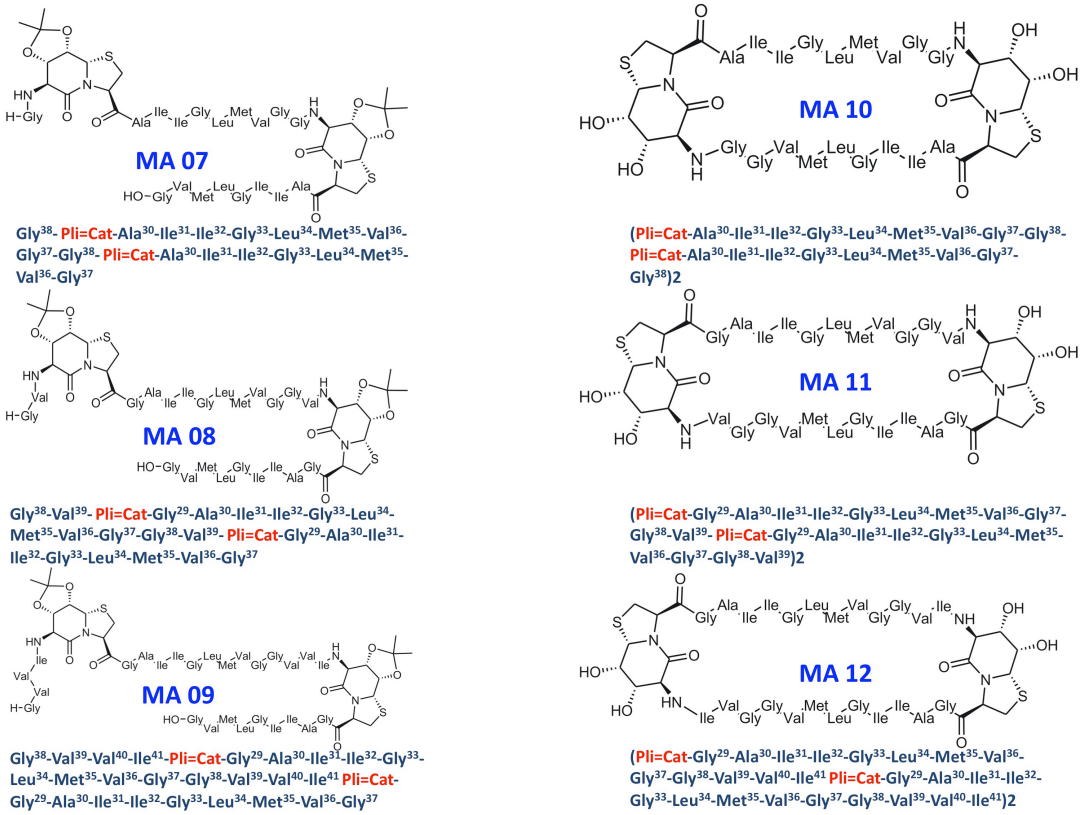

Figure 24 Chemistry of MA peptides 07-12

The MA 07, 08 and 09 are the linear precursors of MA 10, 11 and 12 cyclic peptides. They are flexible dimers of the fragments $A\beta_{30-38}$, $A\beta_{29-39}$, $A\beta_{29-41}$ and in their attributes similar to MA-02. The cyclic peptides MA-10, -11 and -12 are related to MA-06. The backbone of the peptides was directly incorporated with a more rigid linker. The linker 6,5-Bicyclic thiazolidine lactam (Pli=cat) is a β-turn mimetic, guiding the conformation of the peptide towards a β-sheet.

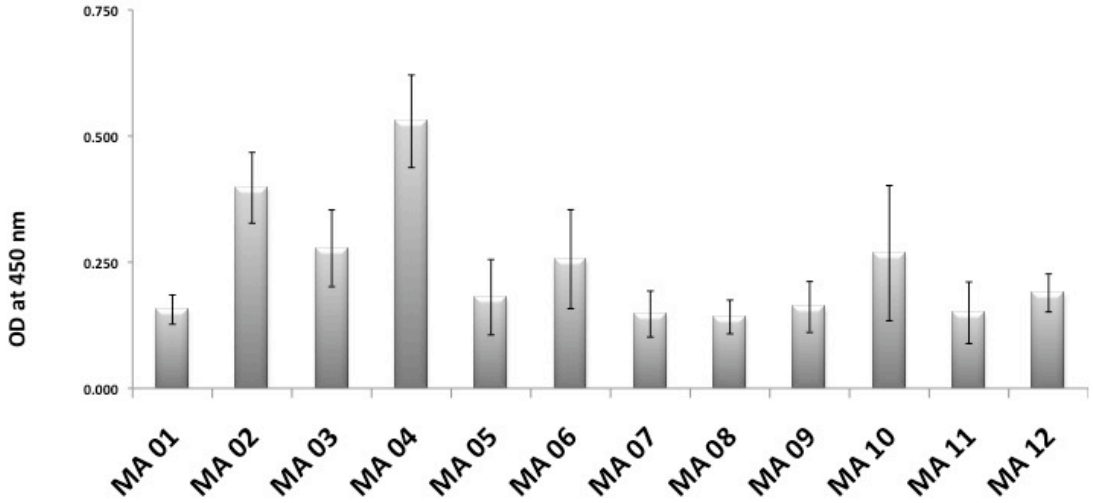

Figure 25 Comparison of NAbs-Aβ binding towards MA 01 to 12. Bars represent the optical density at 450, measured using indirect ELISA, an index of NAbs-Aβ binding toward MA peptides. Number codes represent different MA peptides used in the experiments. Figure represents data from one of the three dilutions used and results from three independent experiments.

Data showed that NAbs-Aβ recognize MA 02 and MA 04 better than the rest of the peptides compared (Figure 25). In agreement with the previous epitope characterization, addition of hydrophobic amino acid at the 33rd position of $A\beta_{28\text{-}40}$, in this case t-leucine inclusion improved the NAbs-Aβ binding towards MA 04. The open-chained parallel dimers MA 02 stood as second best peptide recognized by NAbs-Aβ. Apart from these two, NAbs-Aβ binding was moderate towards the $A\beta_{28\text{-}40}$ sequence containing MA 03 ($A\beta_{28\text{-}40}$) and MA 06. The peptide sequences that lack lysine at 28th position of Aβ showed relatively lower binding except MA 10. MA peptides with dimer inducing conformations MA 07, 08 and 09 showed poor binding. Interestingly, anti-parallel, dimeric closed ring structures MA 06 and MA 10 showed improved binding. In contrast, MA 07, the precursor (during peptide synthesis) of MA 10 and has open N and C terminal ends (open ring) showed poor binding (Figure 25).
In summary, results indicated that lysine-containing click peptides and peptides with fixed dimeric alignment were bound efficiently by NAbs-Aβ. .

3.3.1.2 Lysine at 28th position is crucial for NAbs-Aβ binding

Further, truncated Aβ sequences displaying the epitope region (Ile 32) and anti-parallel dimer alignment of the epitope region were synthesized. MA peptide 13 was synthesized exhibiting the epitope region as a short-peptide of 5 amino acids length. MA 14 with same sequence as MA 13 with an additional click peptide 11-Azido-tetraethylenglycol-1-acetid acid (Ata) was synthesized. MA 15, an anti-parallel closed-ring structure with the same sequence as 13 was included in the experiment. Aβ truncated sequence between glycine 29 to isoleucine 32 with an additional isoleucine at position 33 was also included. Aβ sequence running from alanine 30 to glycine 38 with an additional Azp at the N-terminal end was included to induce further oligomerization. MA 18, 19 and 20 with $A\beta_{28\text{-}40}$ with N-terminal Spe, C-terminal Ata and N-terminal Spe along with C-terminal Ata, respectively, which improves oligomerization were included (Figure 26). The binding efficiency of NAbs-Aβ toward the MA peptides 13-20 was compared. Data showed that the peptides lacking Lysine at 28th position of Aβ were not bound by the NAbs-Aβ despite the anti-parallel dimer conformation (Figure 27). This data supports the previous observation with truncated peptides (Figure 10) where lack of Lys28 reduced the binding ability of NAbs-Aβ. On the other hand, MA 20 with both N-terminal Spe and C-terminal Ata improved the NAbs-Aβ binding. The results suggested that the short sequences with 5 amino acids including the epitope region (isoleucine 32) was not enough to be bound by NAbs-Aβ. Addition of oligomerization inducing click peptides or the anti-parallel alignment did not help NAbs-Aβ binding.

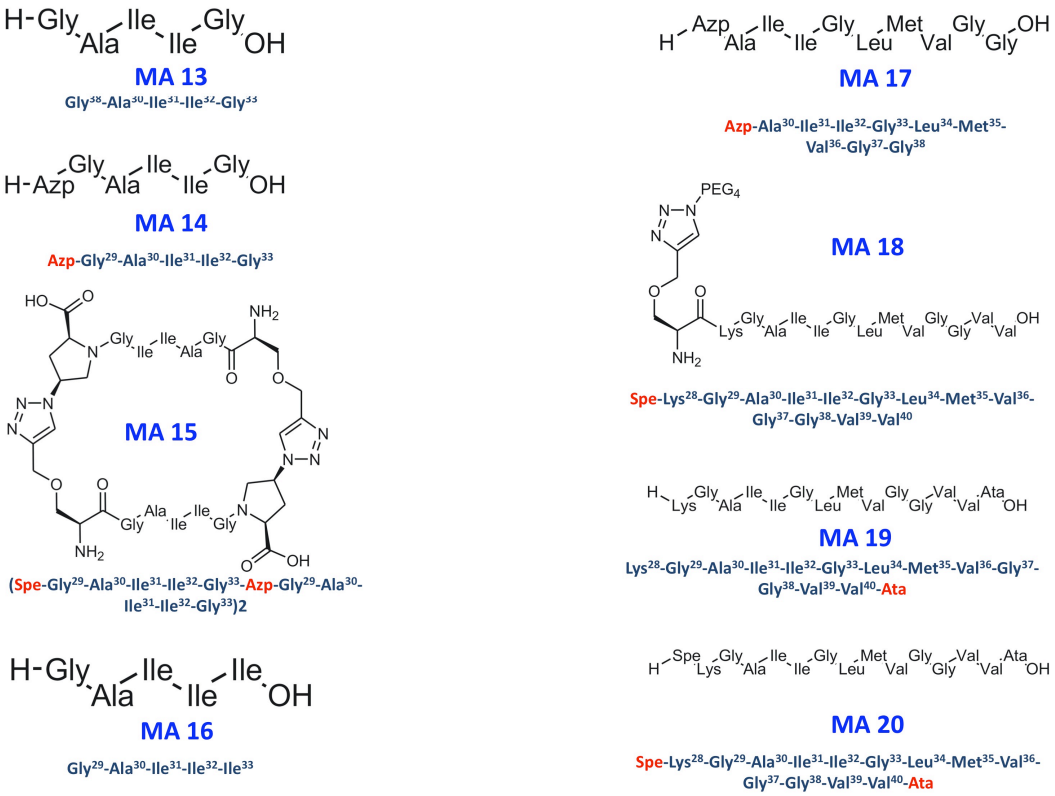

Figure 26 Chemistry of MA peptides 13-20

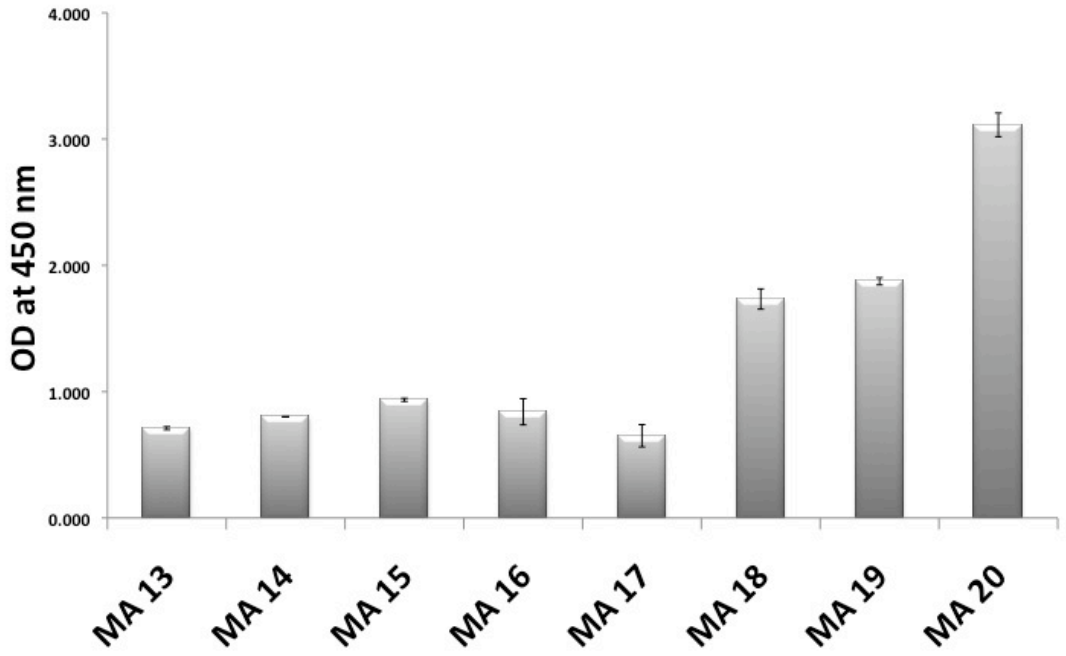

Figure 27 Comparison of MA 13 to 20 towards affinity purified NAbs. Bars represent OD at 450 nm. Number codes represent different MA peptides used in the experiments. Figure represents data from one of the three dilutions used and with three consistent repeats showing similar results from three independent experiments.

3.3.1.3 Comparison of NAbs-Aβ binding to promising conformations of MA peptides

Three promising candidates from each of the initial two experiments (MA 02, 04, 06, 18, 19 and 20) were chosen and compared with the MA peptides 21 to 24 to study NAbs-Aβ binding. Additionally, wt $A\beta_{1-40}$ and $A\beta_{1-42}$ were included as positive controls.

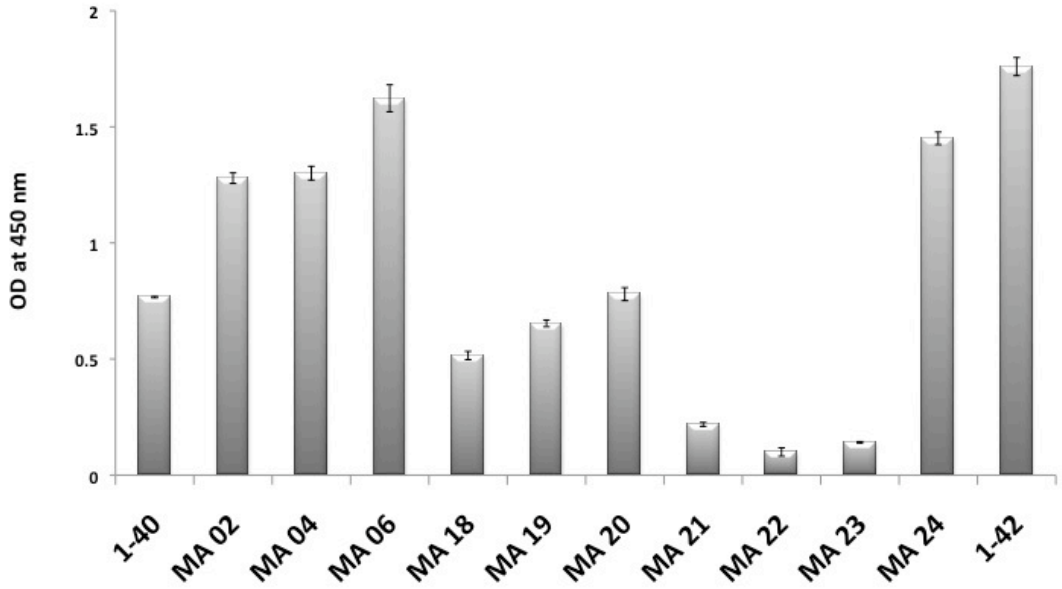

Figure 28 Comparison of best of MA 01to 20, $A\beta_{1-40}$ and $A\beta_{1-42}$ towards affinity purified NAbs. Bars represent OD at 450 nm. Figure represents data from one of the three dilutions used and with three consistent repeats showing similar results from three independent experiments.

MA 21 exhibit $A\beta_{29-33}$ with N-terminal trizol linked Spe (alkine) with α-Azido-Ribose (azide). MA 22 with same sequence as MA 21 with additional C-terminal Ata. MA 23 was designed as closed anti-parallel dimers with click peptide 6-Azido-L-norleucine (Aln) sandwiched between $A\beta_{24-27}$ and glycine 29. MA 24 to 27 had the same sqequence $A\beta_{28-40}$ sequence Spe at the N-terminal and Azp at the C-terminal end forming a ring structure. MA 24-27 differs only in terms of the peptide purity during the preparation (Figure 29).

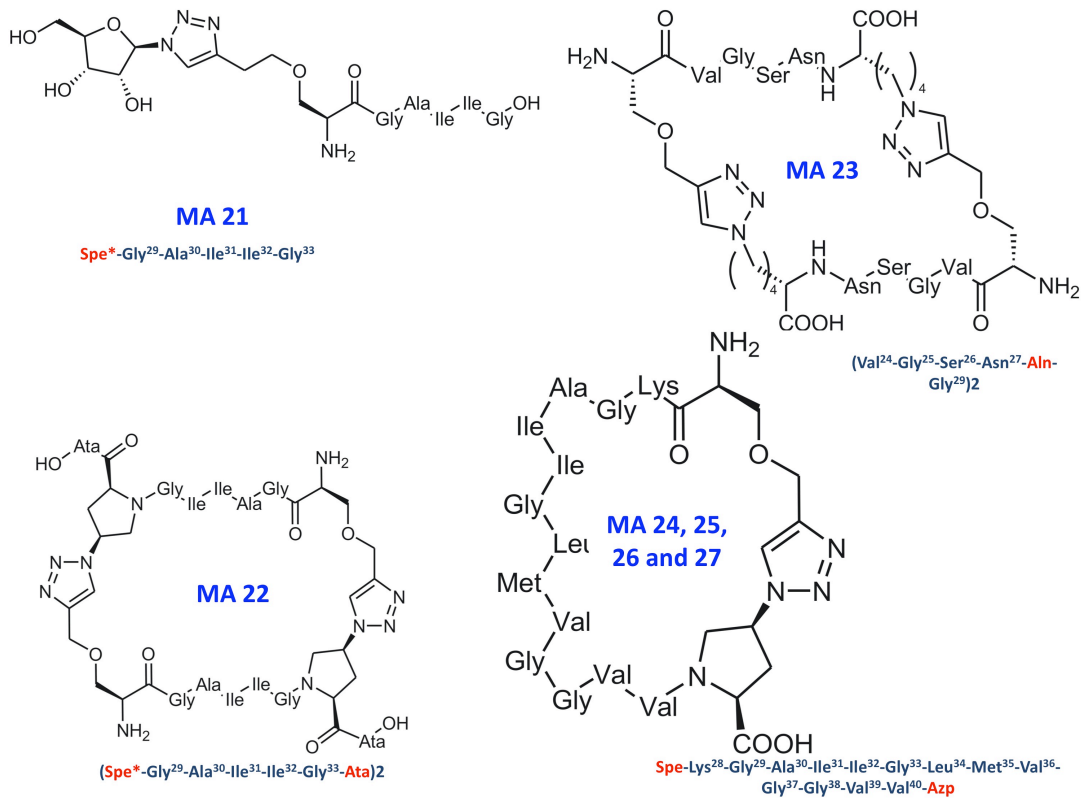

Figure 29 Chemistry of MA peptides 21 to 27

The data demonstrated that MA 06 displaying the epitope sequence with flexible anti-parallel dimeric conformation was recognized by NAbs-Aβ as good as $Aβ_{1-42}$ (Figure 28). MA 24 which exhibit a cyclic structure, MA 02 that has a flexible parallel dimeric conformation and MA 4, a truncated peptide of sequence 28-40 with G33Tle (L-*tert*-Leucine) mutation were recognized better by NAbs-Aβ next to the above mentioned peptides. Fitting to the previous observations, among the positive controls, NAbs-Aβ recognize $Aβ_{1-42}$ better than $Aβ_{1-40}$. Cyclic conformation that represent truncated epitope region between Glycine ^{29} to Glycine ^{33} MA 21 and MA 22 were not exhibiting the epitope conformation bound by NAbs-Aβ despite having the epitope region and cyclic conformation as MA 24. MA 18-20 having 28-40 linear sequence had less binding due to the addition of 11-Azido tetraethylenglycol (AEG_4) in MA 18, 11-Azido-tetraethylenglycol-1-acetid acid (Ata) in MA 19 or L-Serine propargyl ether (Spe) in MA 20.

3.3.1.4 Comparison of C-terminal specific antibodies binding to promising conformations of MA peptides

To make sure that NAbs-Aβ binding towards the peptides is specific and not because of any unspecific molecular crowding effect, Aβ specific monoclonal antibodies were tested as follows. Click peptides used in the previous experiment (3.3.1.3) were tested with C-terminal specific 5C3 and mid-terminal specific 4G8 monoclonal antibodies. The monoclonal antibody 5C3 was chosen due to its unique ability to bind exclusively to C-terminal end of $A\beta_{1-40}$ but not towards $A\beta_{1-42}$. Additionally, mid-terminal antibody (4G8) was used as a negative control in the experiment. MA peptides possess the C-terminal sequence of Aβ and lack the mid-terminal domain toward which 4G8 antibodies are directed at. As a positive control to the full length sequence of $A\beta_{1-40}$ and $A\beta_{1-42}$ were included in the experiment.

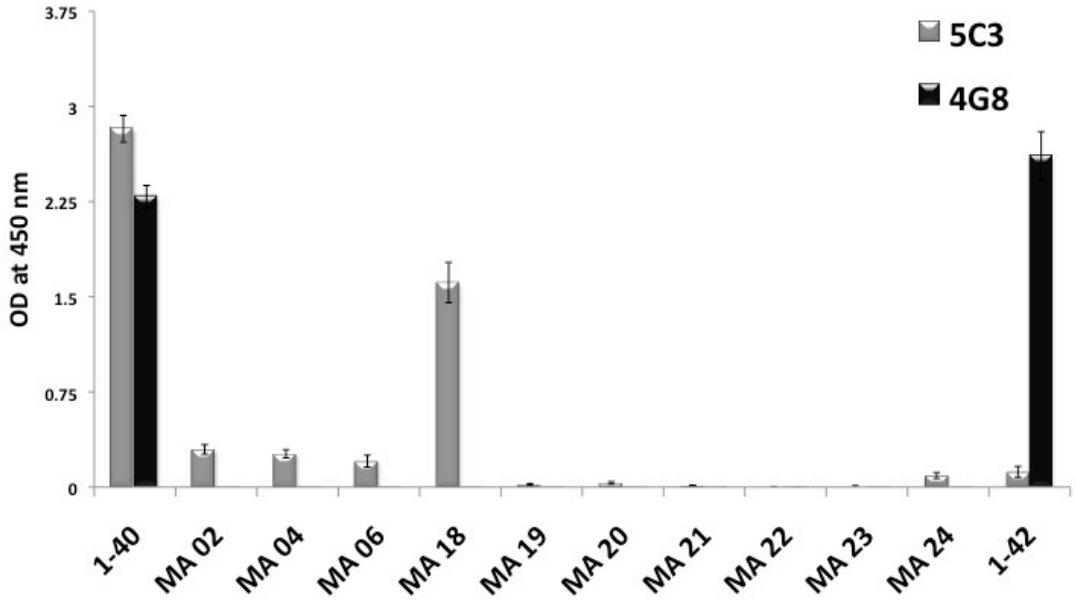

Figure 30 Comparison of best of MA 01to 24, $A\beta_{1-40}$ and $A\beta_{1-42}$ towards NAbs. C-terminal $A\beta_{1-40}$ specific monoclonal antibody 5C3 and mid-terminal specific m266 were used to probe the plates coated with different peptides. Number codes represent different MA peptides used in the experiments. Bars represent OD at 450 nm, bars in grey and black represent signal obtained due to binding towards peptides using 5C3 and 4G8, respectively. Figure represents data from one of the three dilutions (1:5000 among 1:2500, 1:5000 and 1:10000) with 5C3 and one dilution with 4G8 (1:5000) and four independent experiments.

The results showed the specificity of the 5C3 antibodies binding towards $A\beta_{1-40}$ or truncated forms with unaffected C-terminal domain MA 18 and with least binding towards $A\beta_{1-42}$ and other MA peptides.

The mid-terminal specific 4G8 antibodies detected full length $A\beta_{1-40}$ and $A\beta_{1-42}$ with intact mid-terminal sequence. In contrary, 4G8 does not bind any of the C-terminal domains of $A\beta_{1-40}$ that lacks mid-terminal domain.

3.3.1.5 NAbs-Aβ specifically recognize anti-parallel dimers

In order to precisely find out the conformation of Aβ NAbs-Aβ recognize, as a final comparison, the most promising candidates among the previously compared peptides was studied. Additionally, MA 12 peptide which also displays anti-parallel dimeric structure, and the positive controls $A\beta_{1-40}$ and $A\beta_{1-42}$ were included.

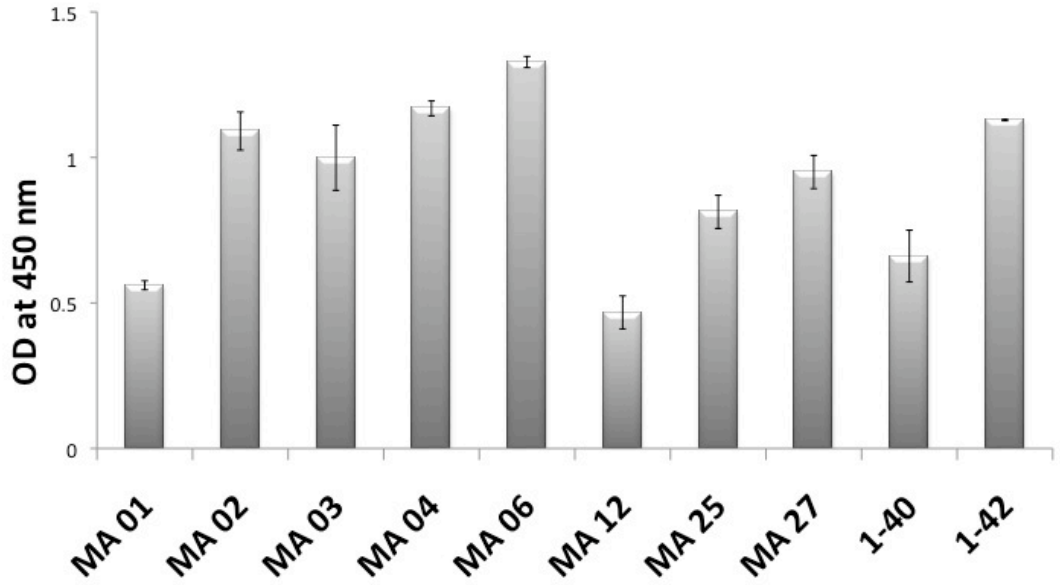

Figure 31 NAbs-Aβ recognize anti-parallel flexible dimeric structure of C-terminus of Aβ. Comparison of best of MA 1 to 25, MA 27, $A\beta_{1-40}$ and $A\beta_{1-42}$ towards affinity purified NAbs. Number codes represent different MA peptides used in the experiments. Bars represent OD at 450 nm. Figure represents data from one of the three dilutions used and three independent experiments.

Compared to the other promising candidates tested in our previous experiments, NAbs-Aβ binding towards MA 6 remained the best (Figure 31), closely followed by MA 02 and MA 04. To some extent MA 03 and MA 25 were also displaying good

signals indicating NAbs-Aβ interaction. $A\beta_{1-40}$ and $A\beta_{1-42}$ showed similar pattern falling in line with our previous observations. MA 12 despite its anti-parallel dimeric structure did not provide a convincing signal, showing NAbs-Aβ recognize flexible anti-parallel dimers.

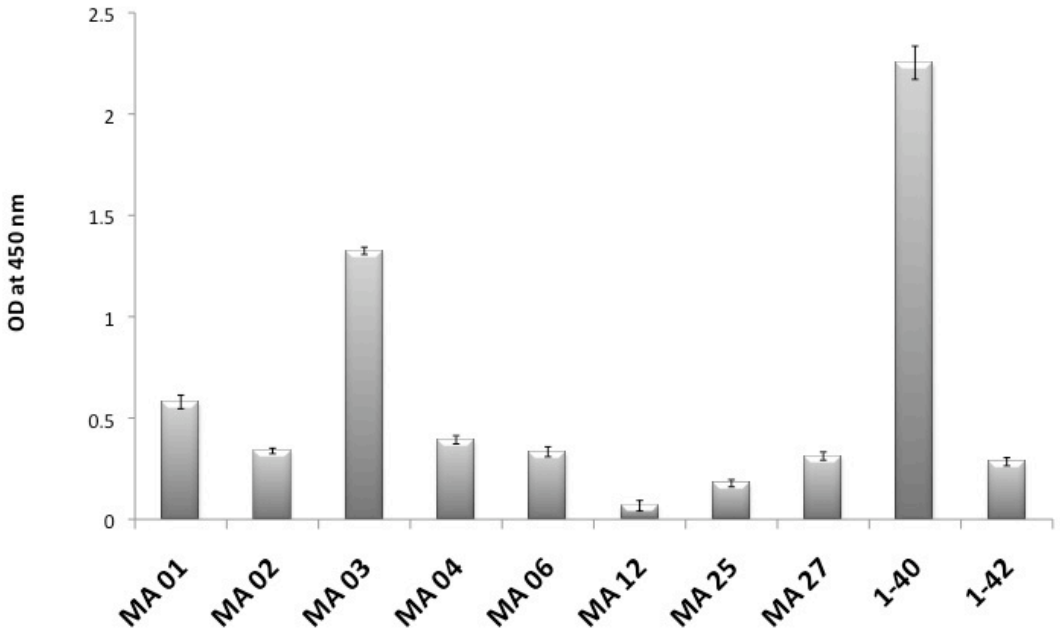

Figure 32 Comparison of best of MA 01to 25, MA 27, $A\beta_{1-40}$ and $A\beta_{1-42}$ towards $A\beta_{1-40}$ C-terminal specific 5C3 monoclonal antibodies. Number codes represent different MA peptides used in the experiments. Bars represent OD at 450 nm. Figure represents data from one of the three dilutions used and with four consistent repeats showing similar results from three independent experiments.

As a control, the same set of peptides was used to study the binding of C-terminal specific 5C3 antibodies. The data showed 5C3 antibodies' specificity towards $A\beta_{1-40}$ and MA 03 which is truncated form of C-terminal sequence 28-40.

In summary, NAbs-Aβ binds to anti-parallel dimers and lysine residue at position 28 helps its binding. Despite the inclusion of anti-parallel structure or the epitope region (isoleucine 32), the results demonstrate that the NAbs-Aβ binding is highly dependent on the anti-parallel structure including the lysine 28.

3.4 Characterization of C-terminal substitution mutant ($A\beta_{1-40;G29,33I}$)

Epitope characterization studies revealed isoleucine substitution of at Aβ positions 29 and 33 improved NAbs-Aβ binding (Figure 14). Further experiments showed the preferential binding of NAbs-Aβ toward oligomers of Aβ. In this regard, the double mutant $A\beta_{1-40;G29,33I}$ were tested for their oligomerization properties, secondary structures and cytotoxicity in the following sub-chapters.

3.4.1 Double mutations at the C-terminal end instantly forms SDS-stable oligomers

To study the oligomerization characteristics of the mutation at the particular sites, freshly dissolved peptides were analyzed using SDS-PAGE. In addition to $A\beta_{1-42;G29,33I}$, two more full length $A\beta_{1-42}$ peptide sequences, $A\beta_{1-42;G29,33I}$ and $A\beta_{1-42;G29,33I;A41I}$, were included in the experiment. $A\beta_{1-42}$ were chosen due to its highly hydrophobic C-terminal sequence which tend to trigger oligomerization.

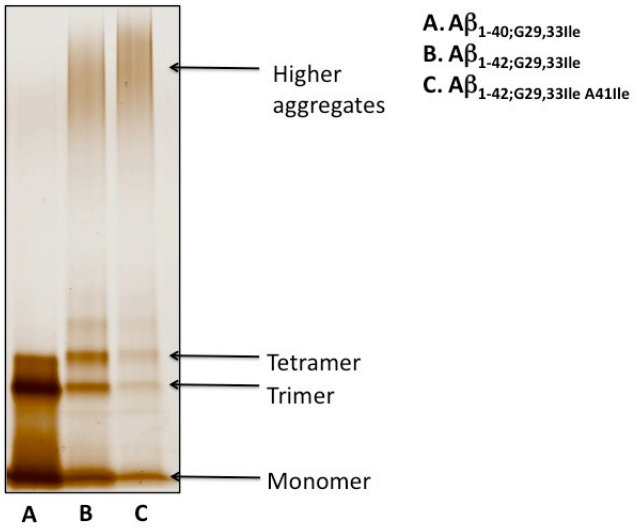

Figure 33 Oligomerization of Aβ due to isoleucine substitution. Silver stained SDS-PAGE show freshly dissolved mutant Aβ instantly form oligomers. Lanes 1, 2 and 3 were loaded with freshly resuspended Aβ mutants $A\beta_{1-42;G29,33I}$, $A\beta_{1-42;G29,33I}$ and $A\beta_{1-42;G29,33I;A41I,}$ respectively.

Silver-stained SDS-PAGE results of mutant peptides $A\beta_{1-40;G29,33Ile}$, $A\beta_{1-42;G29,33Ile}$ and $A\beta_{1-42;G29,33Ile\&A42Ile}$ shows different pattern of oligomerization state. The double

mutation at positions 29 and 33 on $A\beta_{1-40}$, form trimers and tetramers and less higher aggregates. On the contrary, $A\beta_{1-42}$ with the same mutation form higher aggregates in addition to trimers and tetramers. $A\beta_{1-42}$ with triple isoleucine substitution at positions 29, 33 and 41 form similar pattern like $A\beta_{1-42;G29,33I}$.

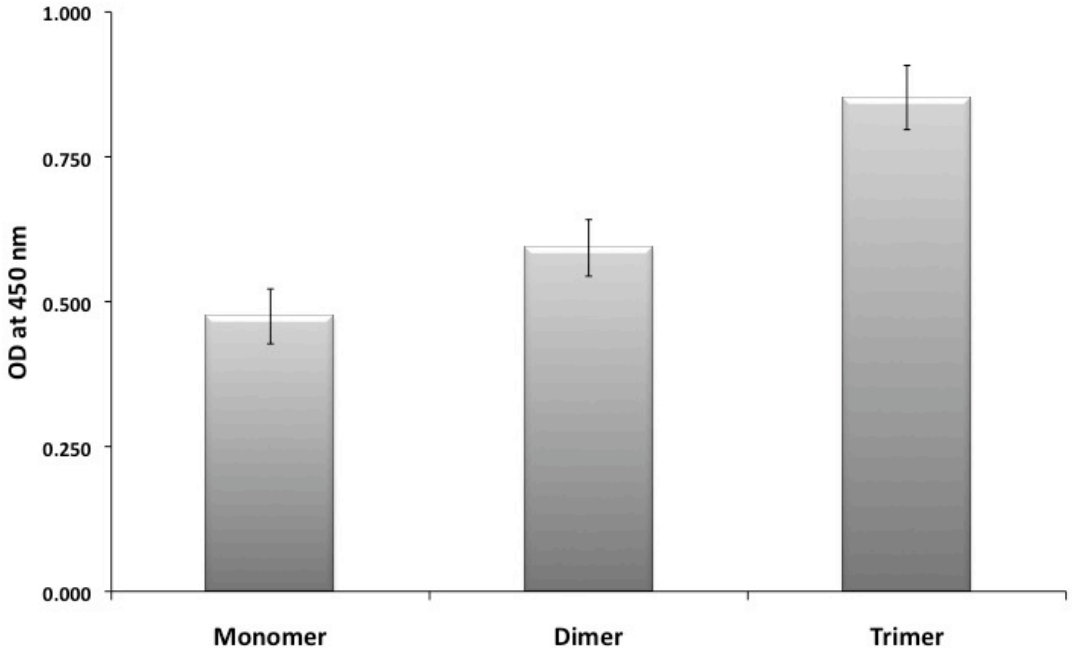

Figure 34 Comparison of NAbs-Aβ binding towards monomers, dimers and trimers. Indirect ELISA, monomeric $A\beta_{1-40}$, dimeric oligomerized Cys-$A\beta_{1-40}$ and trimeric $A\beta_{1-40;G29,31I}$ were coated with 0.1M Sodium Carbonate buffer. Affinity NAbs-Aβ (1mg/mL) were diluted with 1XPBS to 1: 3000 with quadruplicates. Bars represent the optical density measured at 450 nm after the treatment with HRP labelled goat-anti-human secondary antibodies followed by addition of TMB substrate. Figure represents the one of the dilutions among 4 dilutions with consistent pattern in three independent experiments.

3.4.2 Double mutation at the GxxxG region decreases the cytotoxicity of the peptide

The double mutation at GxxxG region of $A\beta_{1-40;G29,33I}$ (G29xxxG33 to I29xxxI33) forms instant SDS-stable trimers (Figure 33). Aβ peptide has been shown to be toxic to the neuronal cells. As $A\beta_{1-40;G29,33I}$ mutant forms instant trimers, its secondary structure, size and conformation of this mutant peptide might also differ from WT Aβ. In this regard further experiments were conducted to analyze the cytotoxicity of Aβ mutants. To study the toxicity, murine microglial and neuronal cells were treated with $A\beta_{1-40;G29,33I}$ mutant and WT $A\beta_{1-40}$ and $A\beta_{1-42}$, subsquently MTT assay was performed

for cell viability. Two different concentrations and different oligomerization states of peptides were used for the toxicity studies. In addition, a non-steroidal anti-inflammatory drug, CNI-1493, which was previously reported to influence in Aβ oligomerization and improves cognition in transgenic animals were also included in the studies (Bacher et al., 2008).

The cytotoxicity assays results from WT peptides were published by Dr. Bach *et al* (Bach et al.). The results shown here are repetition of the similar experimental parameters in addition to mutant peptides. I would like to thank Mr. David Megel for the support he offered by providing the protocol and the cell lines.

3.4.2.1 Monomerized peptides (wild type and mutants) show no neurotoxicity

Murine neuronal cells were treated with 1 or 5 µM of HFIP-pre-treated WT $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides. HFIP treatment breaks down the oligomers into monomers by disrupting the tertiary structures (Kayed et al., 2003). Neuronal cell lines were preferred over the primary neurons as they are easy to cultivate. Additionally, CNI-1493 was included to study the restoration of cell viability exerted by Aβ toxicity.

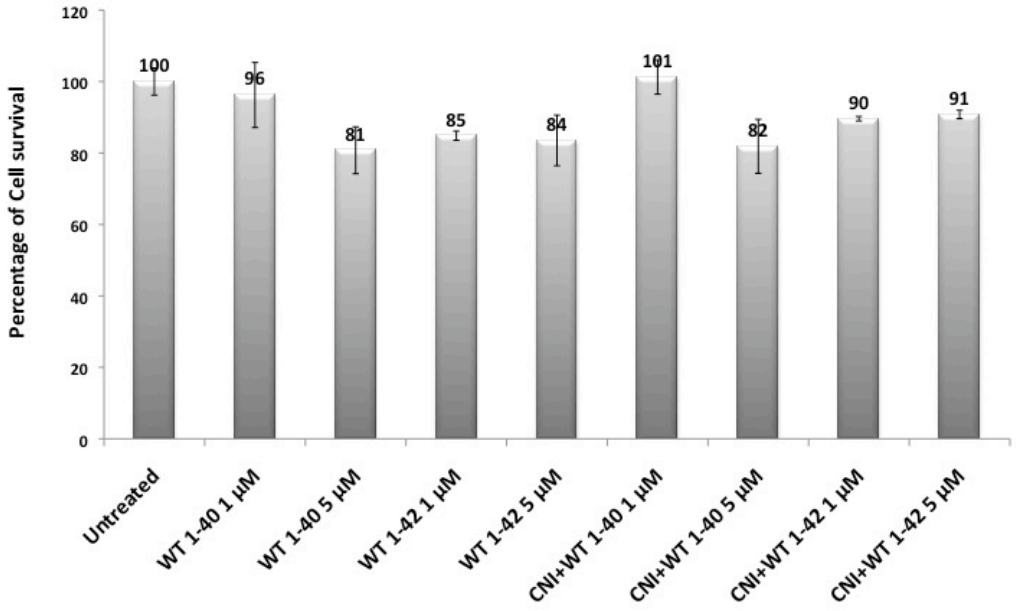

Figure 35 Neurotoxicity of monomerized WT $A\beta_{1-40}$ and $A\beta_{1-42}$; and the role of CNI-1493 in preventing the toxic effect. Murine neuronal cell line N2A were treated for 48 hours with 1 and 5 µM of HFIP-pre-treated $A\beta_{1-40}$ and $A\beta_{1-42}$ which will contain more monomers due to HFIP pretreatment. Optical density corresponding to the MTT level of the untreated cells was set as 100% and percentage

of the cell survival of the Aβ treated was calculated. Bars represent percentage of cell survival after the peptide treatment. The data are representative of duplicated samples and one of three experiments.

Data shows that the treatment of $A\beta_{1-40}$ or $A\beta_{1-42}$ freshly suspended peptides showed minimal toxicity with a maximum of approximately 20% toxicity with $A\beta_{1-40}$ WT at 5 μM concentration when compared to the untreated cells (Figure 35). These results are in line with previous reports where monomeric Aβ showed no neurotoxicity (Kayed et al., 2003).Whereas, CNI-1493 influence on cell survival is also found to be meager.

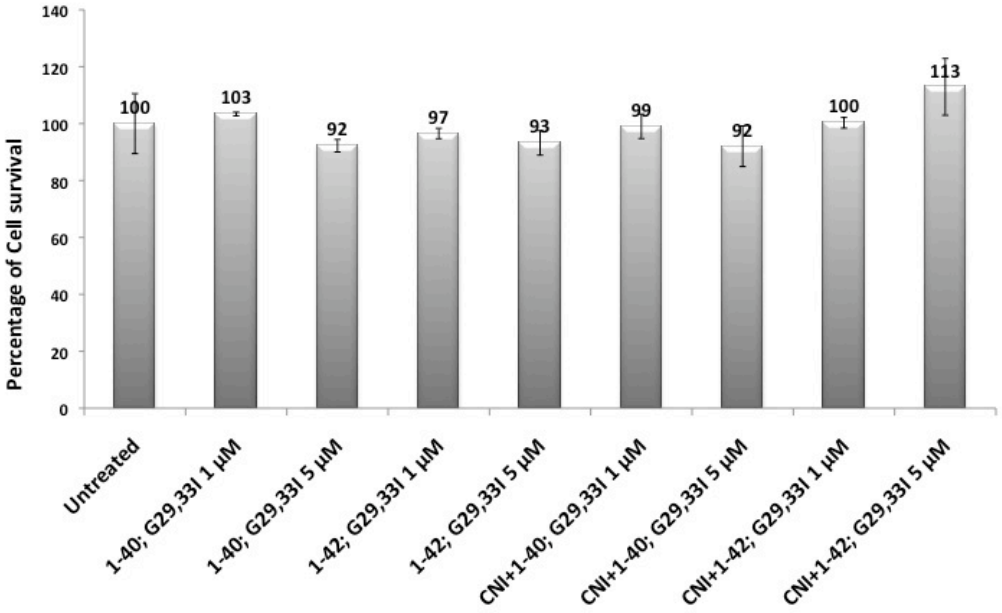

Figure 36 Neurotoxicity of mutants $A\beta_{1-40;G29,33I}$ and $A\beta_{1-42;G29,33I}$. N2A were treated with 1 and 5 μM concentration of freshly prepared $A\beta_{1-40}$ and $A\beta_{1-42}$ monomers for 48 hours. Bars represent percentage of cell survival after peptide treatment. The data are representative of duplicated samples and one of three experiments.

On the other hand, treatment of $A\beta_{1-40;G29,33Ile}$ and $A\beta_{1-42;G29,33Ile}$ freshly suspended peptides showed very less toxicity (Figure 36). This toxicity exerted by mutant peptides are comparitively weaker than the wild type peptides (Figure 35). The effect of CNI-1493 was found to be minimal due to the less toxicity driven by the mutant Aβ.

3.4.2.2 Monomerized Aβ peptides (wild type and mutants) show no toxicity on BV-2 cells

Further, the Aβ toxicity on microglial cells was determined by the treatment of freshly prepared (monomerized) WT $A\beta_{1-40}$ and $A\beta_{1-42}$ of concentrations 1 and 5 µM. Similar to the N2A Aβ treatment, CNI-1493 treatment was also included in the experiment

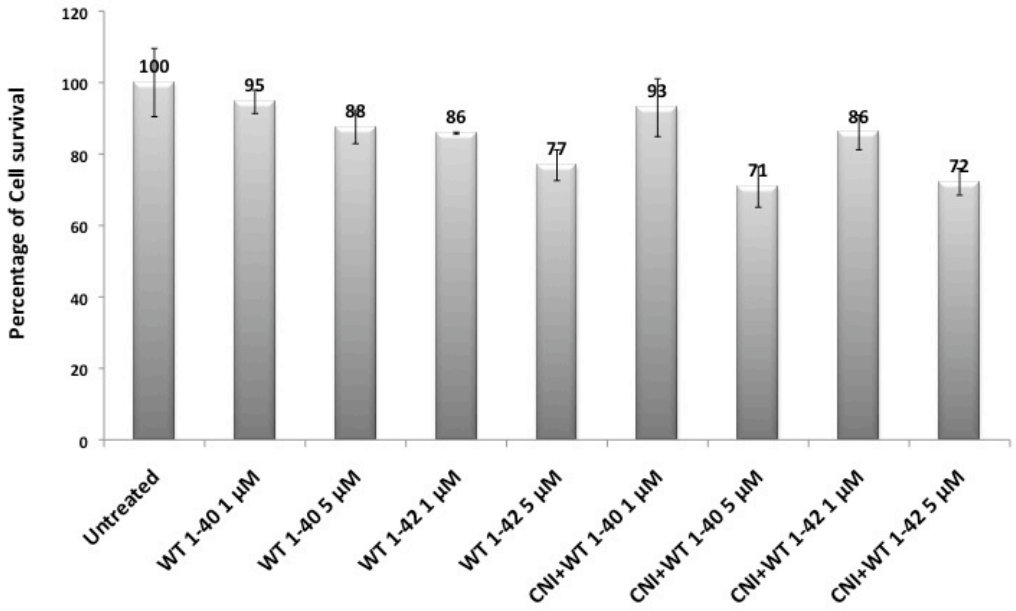

Figure 37 The cytotoxic effect of monomerized $A\beta_{1-40}$ and $A\beta_{1-42}$; and the role of CNI-1493 in microglial cell line (BV-2). Murine microglial cells (BV-2) were treated with freshly prepared HFIP pre-treated $A\beta_{1-40}$ and $A\beta_{1-42}$ monomers. Bars represent percentage of cell survival after peptide treatment, where OD corresponding to the untreated cells were taken as 100% cell survival. The data are representative of duplicated samples and one of three independent experiments.

Treatment of monomerized $A\beta_{1-40}$ and $A\beta_{1-42}$ on microglial cell line showed moderate toxicity. Maximal toxicity of 27% was found in cells treated with WT $A\beta_{1-42}$ at 5 µM concentration (Figure 37). CNI-1493 showed no beneficial effect in improving cell survival but shown a little toxicity, which is a negligible percentage.

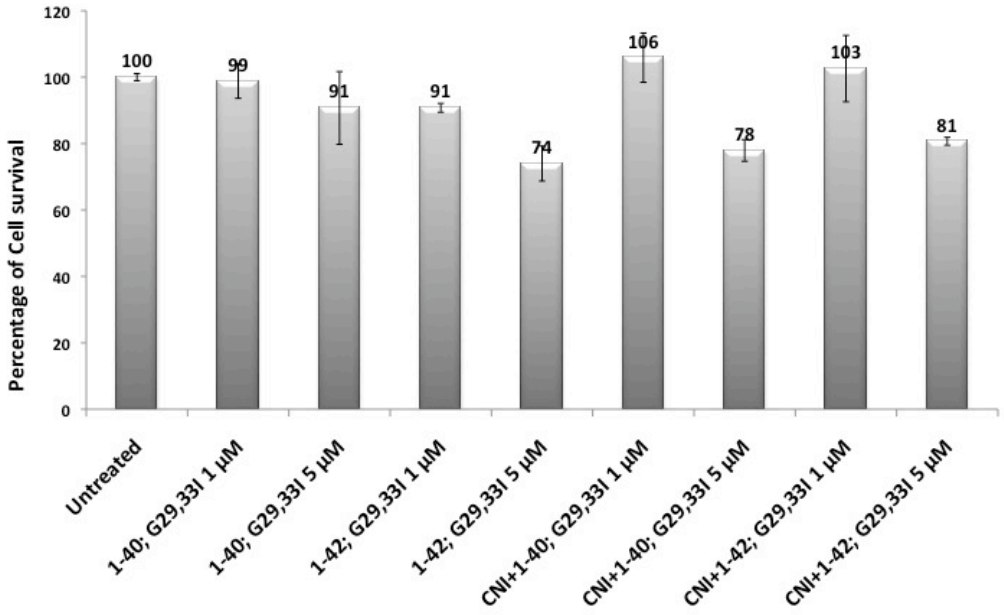

Figure 38 The cytotoxicity effect of mutants $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$. Murine microglial cells were treated with 1 and 5 µM concentration of HFIP pre-treated mutant peptides $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$. Bars represent percentage of cell survival after peptide treatment, where OD value of untreated cells were taken as 100% cell survival. The data are representative of duplicated samples and one of three experiments.

Mutant peptide treatment did not affect the BV-2 cell survival except 5 µM $A\beta_{1\text{-}42;G29,33I}$, which showed approximately 26% toxic, compared to the untreated cells. CNI-1493 showed no beneficial effect on peptide treated with Aβ mutants (Figure 38).

3.4.2.3 Wild type Oligomers are toxic on N2A cells, and CNI-1493 facilitates the cell survival

It was previously shown that synthetic Aβ oligomers have neurotoxic effect (Kayed et al., 2003). The same Aβ oligomerization protocol was used to study the cytotoxic effect of WT and mutated Aβ. In addition to the peptides, subsequently after Aβ oligomers preparation peptides were co-incubated for 20 mins with CNI-1493. Similar to the previous experiments, neuronal cell line N2A was used to perform the MTT cell viability assay.

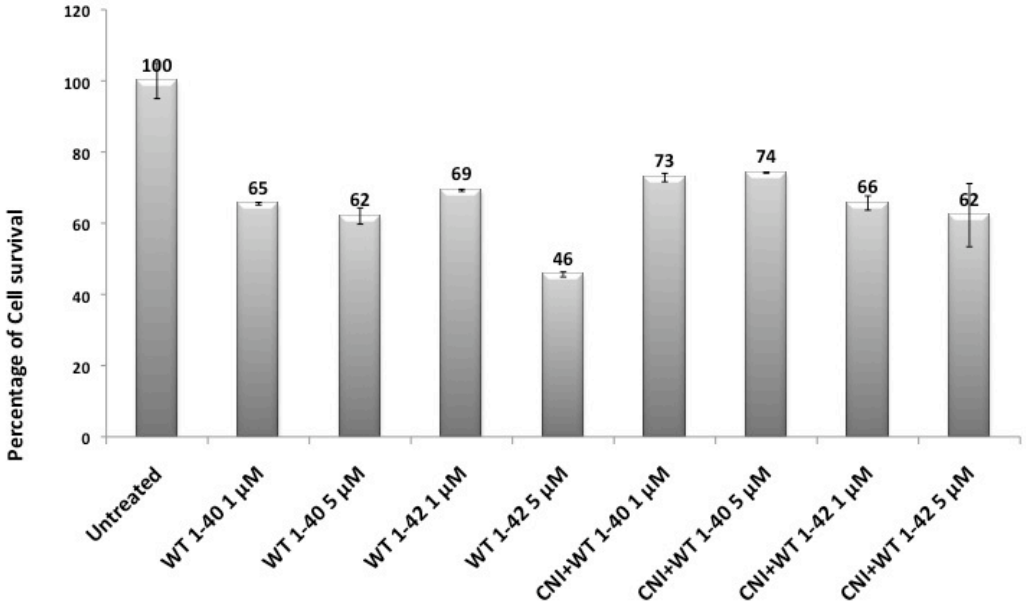

Figure 39 Neurotoxicity of oligomers of wild type $A\beta_{1-40}$ and $A\beta_{1-42}$; and the rescuing effect CNI-1493. N2A were treated with oligomers of WT $A\beta_{1-40}$ and $A\beta_{1-42}$. Bars represent percentage of cell survival after peptide treatment where OD of untreated cells were taken as 100%. The data are representative of duplicated samples and one of three experiments.

In contrary to monomerized Aβ, treatment of oligomerized both $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides showed toxicity on neuronal cell lines. Oligomers of $A\beta_{1-40}$ showed approximately 35 to 38% toxicity at 1 and 5 µM concentrations respectively. Concentrations were calculated according to the amount monomers of Aβ, as the exact proportion of Aβ species could not be accurately estimated due to its high aggregation rate. Oligomeric species of $A\beta_{1-42}$ exerted more toxicity on neuronal cells compared the $A\beta_{1-40}$ oligomers. 1 µM $A\beta_{1-42}$ oligomers induced approximately 27% of cell death and remarkably 5 µM triggered 54% toxicity (Figure 39).

CNI-1493 treatment improved the cell survival from both $A\beta_{1-40}$ and $A\beta_{1-42}$ induced toxicity. The treatment showed notable improvement in cell survival from olgomeric $A\beta_{1-42}$ induced toxicity.

3.4.2.4 Oligomers of mutant peptides are non-toxic to N2A cells

Treatment of wt Aβ oligomers showed to be toxcit to the neuronal cells (Figure 39). To determine the toxicity of mutant Aβ peptide, $A\beta_{1-40;G29,33I}$ and $A\beta_{1-42;G29,33I}$ were

separately incubated under the same oligomerization conditions as the previous experiment (3.4.2.3).

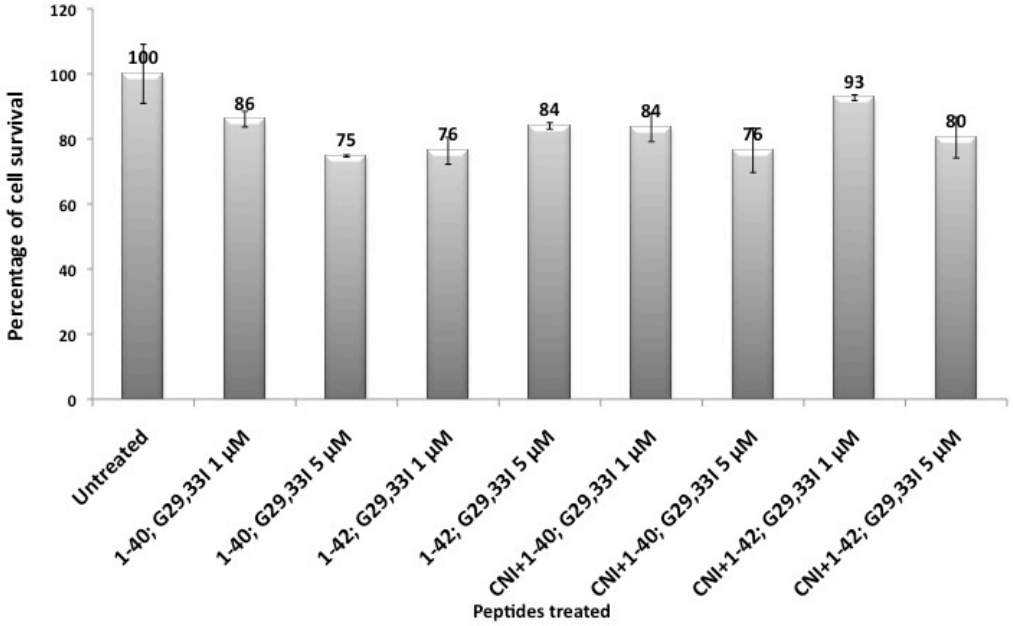

Figure 40 The Neurotoxicity of oligomers of mutant $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$ and the role of CNI-1493 in preventing the toxic effect. N2A were treated with $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$ pre-incubated under oligomer inducing conditions. OD obtained with untreated cells were set as 100% cell survival. Bars represent percentage of cell survival calculated with OD of untreated cell survival as 100%. The data are representative of duplicated samples and one of three experiments.

In this approach, oligomers of $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$ did not show as much toxicity as WT oligomers. 1 and 5 µM $A\beta_{1\text{-}40;G29,33I}$ exerted 14 and 25 %, respectively. Whereas, 1 µM of $A\beta_{1\text{-}42;G29,33I}$ oligomers showed 24% toxicity in contrary 5 µM of $A\beta_{1\text{-}42;G29,33I}$ oligomers showed only 16% (Figure 40). These data suggests that either the mutated Aβ peptides does not form toxic oligomers under the oligomerization condition as WT Aβ does, or oligomers of mutated Aβ are non-toxic. Addition of CNI-1493 shows no remarkable difference in recovering the little toxicity induced by mutant peptides.

3.4.2.5 Wild type Oligomers are toxic on BV-2 cells and CNI-1493 minimizes the toxicity

Similar experiments were performed on BV-2 cells using the oligomers prepared for the N2A cells treatment. WT Aβ oligomers were incubated either with or without CNI-1493 pre-incubation.

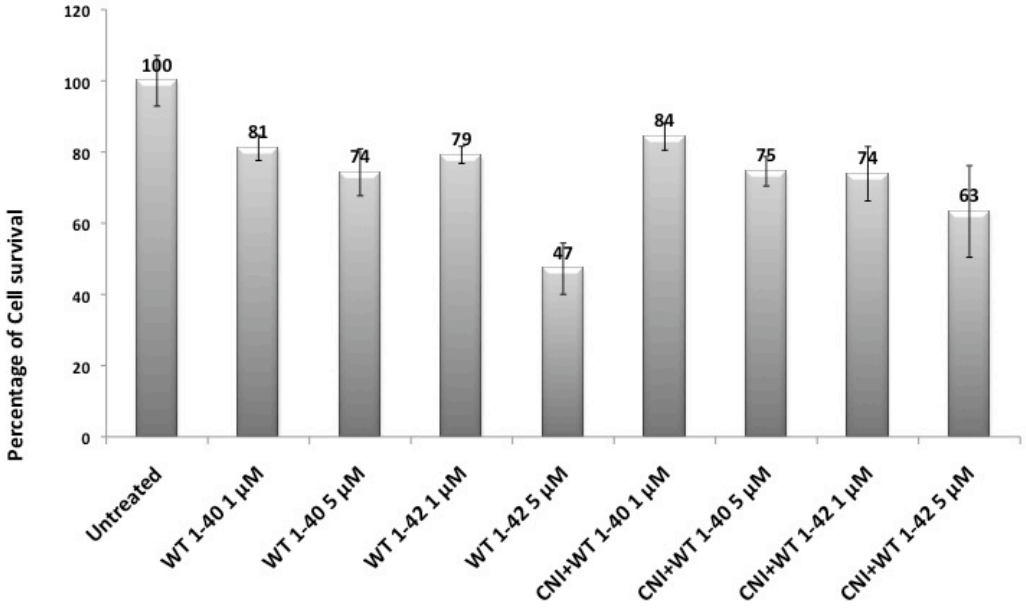

Figure 41 The cytotoxic effect of oligomers derived from wild type $A\beta_{1-40}$ and $A\beta_{1-42}$; and the protective role of CNI-1493 on microglial cell survival. BV-2 cells were treated for 48 hours with oligomers of wild type $A\beta_{1-40}$ and $A\beta_{1-42}$. Prior to treatment on cells the peptides were maintained under conditions which trigger oligomerization. OD values of untreated cells were taken as the 100% surival. Bars represent percentage of cell survival after peptide treatment. The data are representative of duplicated samples and one of three experiments.

Similar to the effect on neuronal cell lines (Figure 39), treatment of oligomerized $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides showed toxicity on microglial cell lines (Figure 41). Treatment of oligomerized $A\beta_{1-40}$ at concentrations 1 and 5 µM showed a moderate toxicity of 19 to 26%, respectively. Microglial cells treated with 1 µM $A\beta_{1-42}$ oligomers induced 21% of cell death and remarkably 5 µM triggered 53% toxicity on microglial cell line. CNI-1493 treatment improved the cell survival from both $A\beta_{1-40}$ and $A\beta_{1-42}$ induced toxicity. The CNI-1493 treatment showed an improvement of around 16% in cell survival against oligomeric $A\beta_{1-42}$ induced toxicity. These results indicate that CNI-1493 has beneficial effect against Aβ toxicity on both neuronal (Figure 39) and microglial cells (Figure 41).

3.4.2.6 Oligomers of mutant peptides are non-toxic BV-2 cells, and CNI-1493 increases the toxicity

Further, the effect of mutant Aβ incubated under oligomerization condition on microglial cells was studied. $A\beta_{1-40;G29,33I}$ and $A\beta_{1-42;G29,33I}$ incubated in an oligomer

inducing condition coupled with or without CNI-1493 co-incubation were fed to the microglial cells.

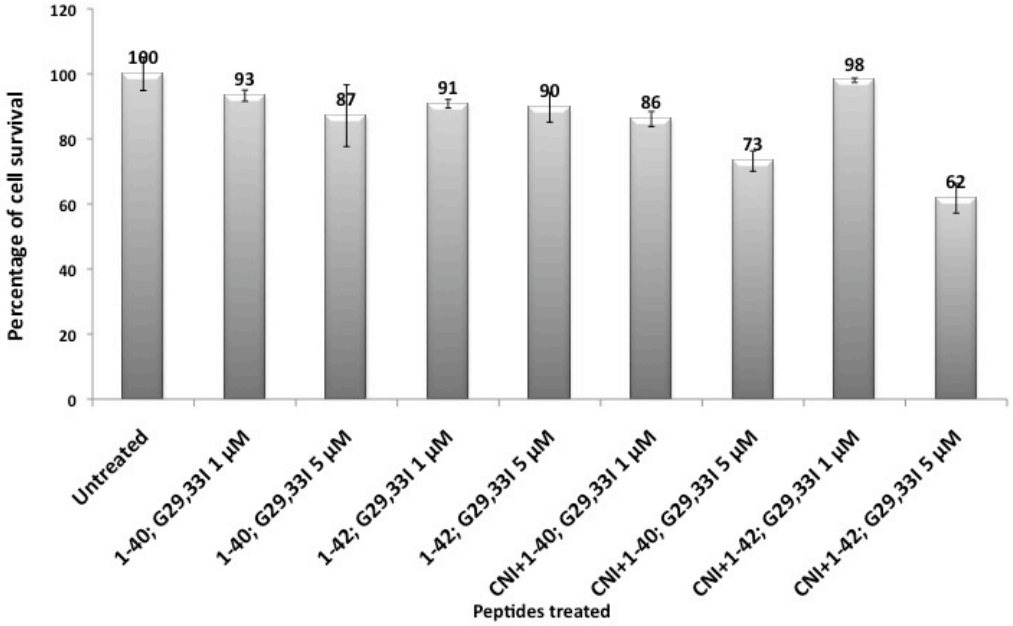

Figure 42 The cytotoxicity of oligomers of mutant $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$ and the role of CNI-1493 treatment. Murine neuronal cells (BV-2) were treated with oligomers of $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$. Prior to treatment on cells the peptides were maintained under inducing conditions with or without CNI-1493. Untreated cells were taken as positive control with 100% cell survival. Bars represent percentage of cell survival after peptide treatment. The data are representative of duplicated samples and one of three experiments.

Treatment of peptides incubated in oligomerization condition on microglial cells showed moderate toxicity. $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$ either undergone monomerizing or oligomerizing treatment did not display any cytotoxic effects on microglial cells. The maximum toxicity exererted by 5 µM $A\beta_{1\text{-}40;G29,33Ile}$ was around 23% (Figure 42). 5 µM concentration of $A\beta_{1\text{-}42}$ double mutant showed even lesser toxicity of only 10 % cell death. On the contrary, addition of CNI-1493 showed 14% and 28% more toxicity than with the cells treated with $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$ alone.

In summary, monomerized WT $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ or mutated $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$ did not show any remarkable toxicity on both neuronal and microglial cells. Co-incubation of monomerized Aβ peptides with CNI-1493 did not offer any beneficial effect. WT $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ incubated under oligomerization inducing condition exerted a noticeable cytotoxicity on neuronal and microglial cell lines. CNI-

1493 restored the cell survival against the WT Aβ oligomers induced cyotoxocity in both cell types. Mutated $A\beta_{1\text{-}40;G29,33I}$ and $A\beta_{1\text{-}42;G29,33I}$ did not show any toxicity in both monomerized or incubated in an oligomer inducing condition on any of the cell type. On the contrary, microglial cells treated with mutant Aβ peptides co-incubated with CNI-193 in an oligomer inducing condition showed a moderate cytotoxicity.

3.5 Comparison of amyloid beta specific antibodies to NAbs-Aβ

Built around Aβ hypothesis, there are clinical trials involving Aβ immunotherapy for AD. Aβ form several intermediates between monomers to fibrils. While targeting the Aβ it is crucial to know the toxic species, as Aβ is important for normal synaptic functions. Recent evidences suggest that Aβ oligomers are causative agents of Aβ, hence, targeting Aβ oligomers are expected to improve the rate of success in AD therapy. In this regard, this study was planned to study the antibodies that are currently in clinical trial to understand their binding properties toward Aβ oligomers. In the previous chapters, a mutant Aβ oligomer that stabilizes a trimeric conformationally distinct Aβ (3.4.1) and a stabilized dimers using cysteine were characterized (3.2.1). The monoclonal antibodies currently undergoing clinical trials and NAbs-Aβ were compared for their binding toward different oligomeric species of Aβ. In the first experiment, binding ability of N-terminal specific murine 3D6 antibodies (Schenk et al., 1999), oligomer specific rabbit A11 antibodies (Kayed et al., 2003) were compared with NAbs-Aβ. In the second experiment, the anti-fibrillation properties of m266 and NAbs-Aβ were tested. 3D6 and A11 antibodies were omitted for the experiments due to their N-terminal specificity that might not influence fibrillation and lack of enough quantity to perform the experiments, respectively.

3.5.1 Comparison of monoclonal antibodies and NAbs-Aβ for Aβ oligomer binding

Antibodies currently in the passive immunotherapy clinical trials are designed to target Aβ. Since the concept of Aβ oligomers was relatively new, and due to lack of stable oligomeric Aβ preparation strategies most of the antibodies in the clinical trial are conformationally unspecific. In this regard, N-terminal specific, conformationally unspecific 3D6, conformational specific A11 antibodies were compared with NAbs-Aβ. In this study, monomerized WT $Aβ_{1-40}$ and $Aβ_{1-42}$, N-terminal cysteine added dimer enriched cys-$Aβ_{1-40}$ (3.2.1) and trimeric $Aβ_{1-40;G29,33I}$ (3.4.1) were immunoprecipitated with the antibodies.

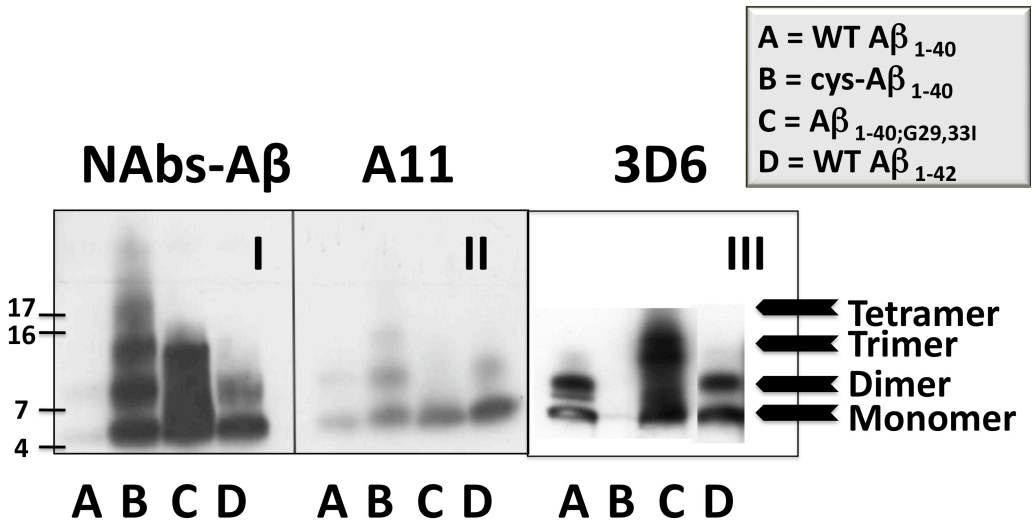

Figure 43 Immunoprecipitation of different Aβ species with NAbs-Aβ, A11 and 3D6 antibodies. Synthetic Aβ peptides in different states of oligomerization were immunoprecipitated using affinity purified NAbs-Aβ, oligomer specific A11 and N-terminal specific 3D6 antibodies. Immunoprecipitates were separated by 10-20% gradient tricine SDS-PAGE. Immunoblots were probed using immunoblotting with two antibodies 6E10 and 4G8, N-terminal and mid-terminal specific antibodies, respectively. 4 peptide preparations immunoprecipitated were: WT $A\beta_{1-40,}$ dimer enriched N-terminally cysteine tagged $A\beta_{1-40}$, double mutant which forms trimers $A\beta_{1-40;G29,33I}$ and WT $A\beta_{1-42}$. Panel I, II and III represents immunoprecipitates of NAbs-Aβ, A11 and 3D6 antibodies, respectively.

Immunoprecipitation experiments (Panel I) indicated that NAbs-Aβ do not recognize monomeric $A\beta_{1-40}$, but could interact to enriched dimers $A\beta_{cys1-40}$, trimeric $A\beta_{1-40;G29,33I}$ and wild type $A\beta_{1-42}$. Results demonstarated that A11 (Panel II) less binding to most of the peptides used. Similar to NAbs-Aβ, A11 does not bind to monomeric $A\beta_{1-40}$ and moderate binding toward $A\beta_{1-42}$. Interestingly, there was low binding towards $A\beta_{1-40;G29,33I}$ mutant peptides, but not to the SDS-stable trimeric $A\beta_{1-40;G29,33I}$. Binding of A11 towards the cysteine containing dimer enriched $A\beta_{cys1-40}$ and WT $A\beta_{1-42}$ were better than with other peptides. 3D6 antibodies bind to most all the species of Aβ, including the oligomers of $A\beta_{1-40;G29,33I}$. The binding of 3D6 toward dimer enriched $A\beta_{cys1-40}$ was found to be affected completely due to the N-terminal cysteine (Figure 43). These results suggested that NAbs-Aβ binding toward the $A\beta_{1-40;G29,33I}$ is not due

to molecular clouding as A11 does not bind to the trimeric species of Aβ. 3D6 demonstrated high specificity toward the N-terminal sequence, incorportation of one additional amino acid completely abolishes the binding. Oligomer specific A11 antibodies showed their specificity toward the oligomeric specific of Aβ. Interestingly, A11 does not recognize the SDS-stable $A\beta_{1\text{-}40;G29,33I}$ but does bind to the SDS-instable $A\beta_{1\text{-}40;G29,33I}$.

3.5.2 NAbs-Aβ prevents fibrillation efficiently compared to m266

To investigation whether NAbs-Aβ could prevent Aβ aggregation, Aβ fibrillation assay was conducted. As there are no widely accepted methods to study the oligomerization, fibrillation assay was used in this regard. NAbs-Aβ, which is C-terminal sequence, and mid-terminus specific m266, were tested for their anti-fibrillation properties. 15 µM antibodies were co-incubated with Aβ to study their efficiency to prevent Aβ fibrillation. $A\beta_{1\text{-}40}$ was used as positive control and taken as 100% value.

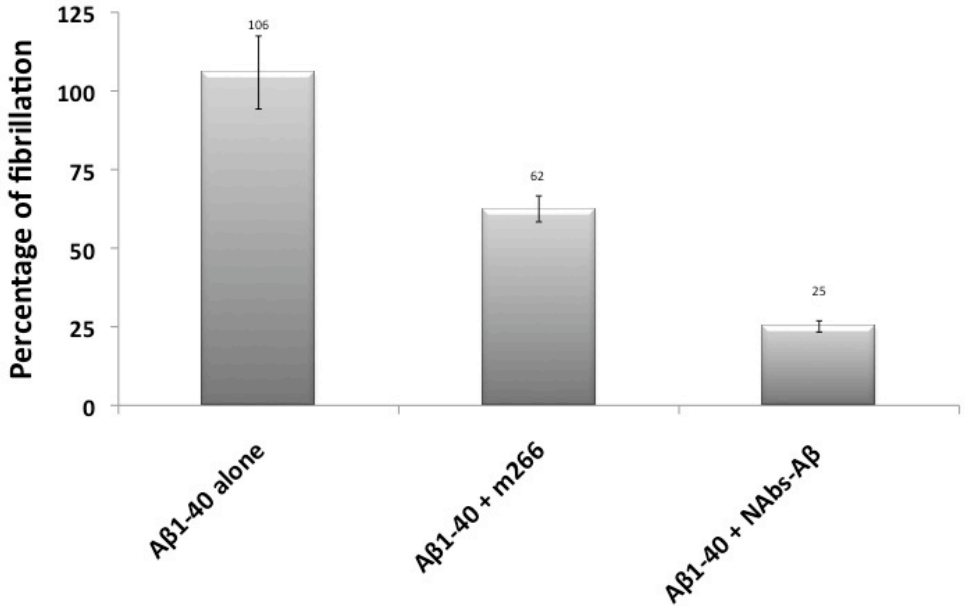

Figure 44 Fibrillation assay: NAbs-Aβ prevents $A\beta_{1\text{-}40}$ fibrillation. $A\beta_{1\text{-}40}$ was incubated with or without antibodies for 48 hours in a fibrillation favouring condition. Fibrillation of $A\beta_{1\text{-}40}$ was taken as 100% and fibrillation in the presence of antibodies were calculated accordingly. The bars represent the percentage of Aβ fibrillation.

The data suggests that NAbs-Aβ could efficiently slow down the fibrillation compared to m266 (**Figure 44**). Compared to the Aβ alone m266 could restrict the fibirllation to 62 % NAbs-Aβ could efficiently supress the fibirllation of Aβ to 25%. This effectively anti-fibrillation property of NAbs-Aβ could be due to its binding specificity toward the C-terminal end of Aβ.

3.6 Effect of an anti-inflammatory (CNI-1493) drug on Aβ oligomers

Alzheimer's disease is a neuro-inflammatory disease and many groups have reported the influence of cytokines in disease progression of this disease. There was more than one clinical trial in which anti-inflammatory drugs had been used for the treatment of Alzheimer's disease. CNI-1493 that is already in clinical phase II research for Corn's disease has MAP-kinase inhibitory action. Previous studies in our laboratory demonstrated that CNI-1493 decreases the plaque deposition and improves cognition in transgenic animals mimicking Alzheimer's disease (Bacher et al., 2008). Previously results supported that the CNI-1493 treatment could exert a protective effect against Aβ oligomer induced toxicity on both neuronal (3.4.2.3) and microglial cell lines (3.4.2.5). In this study, biochemical experiments were conducted to investigate the role of CNI-1493 on oligomers.

3.6.1 CNI-1493 binds to amyloid beta oligomers and breaks them into smaller forms

CNI-1493 showed a scavenger effect on both neuronal and microglial cells treated with Aβ oligomers (Figure 39), (Figure 41). To explore the effect of CNI-1493 on Aβ oligomers, biochemical experiments were performed. Immunoblot and dot blot experiments were conducted on samples, where the CNI-1493 along with Aβ oligomers was co-incubated. Addition of CNI-1493 instantly precipitated Aβ which was visible to the naked eye (Data not shown).

To study the effect of CNI-1493 on the oligomeric Aβ, WT $A\beta_{1-42}$ and $A\beta_{1-42;G29,33I;A41I}$ which forms instant oligomers were co-incubated with or without CNI-1493. Samples were immunobloted and probed with N-terminal specific 3D6 and mid-terminal specific 4G8 antibodies.

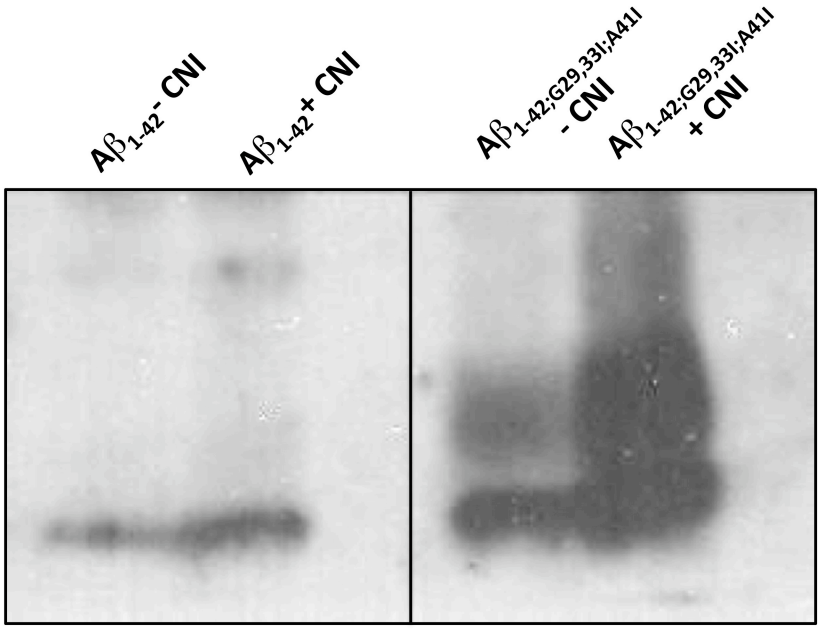

Figure 45 Immunoblot: showing incubation with CNI-1493 breaks the SDS-stable oligomers to yield increased monomer ratio in the treated samples. Oligomers prepared with $A\beta_{1-42}$ wild type and $A\beta_{1-42;G29,33I;A41I}$ mutants were incubated with CNI-1493. The Samples were separated upon their molecular weight by SDS-PAGE gel electrophoresis under non-denaturing conditions using 10-20% tricine gel.The samples were transferred onto nitrocellulose membrane using Western blot and probed using N-terminal speicific 3D6 and mid-terminal specific 4G8 antibodies. In the figure, left panel shows the $A\beta_{1-42}$ and the right panel displays the $A\beta_{1-42;G29,33I;A41I}$ oligomers, incubated without or with CNI-1493.

The results demonstrated that the CNI-1493 could break down the SDS-stable oligomers to monomers (Figure 45). Immunoblotting the samples incubated without CNI-1493 showed less monomers compared to the treated. This effect was also found in $A\beta_{1-42;G29,33I;A41I}$ oligomers treated with CNI-1493, which improves the amount of broken down monomers from the SDS-stable oligomeric complex.

3.6.2 CNI-1493 breaks toxic oligomeric conformation of Aβ

Further, to prove the previous observation, dot blot experiment was conducted. The data provided here are exclusively performed by Mr. Roman Sankowski, Department

of Neurology, Philipps-University of Marburg. I would like to thank him for his gratitude to provide this results to add in my thesis.

Aβ oligomers were co-incubated with different concentrations of CNI-1493 and dotted on nitrocellulose membrane. Blot was then probed using oligomer specific antibodies A11. A11 antibodies as mentioned earlier, recognize exclusively the toxic oligomeric conformation of the Aβ peptide (Kayed et al., 2003). The bound antibodies were removed using stripping procedure and reprobed with N-terminal specific 3D6 antibodies. Stripping and reprobing of the membrane was performed as a positive control to check the presence of Aβ on the membrane.

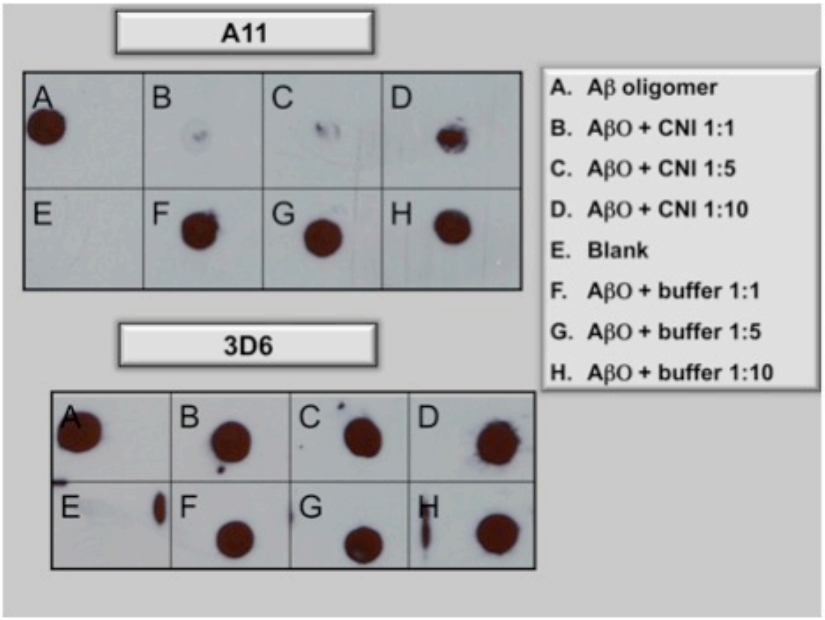

Figure 46 Dot blot demonstrating CNI-1493 affects the conformation of Aβ. Incubation of different concentrations of CNI-1493 shows a concentration dependent decrease in A11 detection (B, C and D), which was not found on the spots where Aβ oligomers were incubated in the solven alone (F, G and H). Stripping the blot and reprobing with N-terminal specific 3D6 antibodies could detect Aβ peptides dotted on the membrane.

Dot blotting with A11 antibodies showed that CNI-1493 destabilizes the conformation of oligomerized Aβ peptides in a concentration dependant manner. The inability of A11 antibodies in the samples coincubated with CNI-1493 proves that the drug could break down the oligomeric conformation of Aβ. Stripping the membrane and reprobing with N-terminal specific 3D6 antibodies was used as a positive loading control. Probing with 3D6 showed the presence of Aβ peptide. As a negative control samples treated with the solvent alone did not affect the binding of both A11 and 3D6 antibodies (Figure 46).

4 DISCUSSION

It is its 'nature' to cause diseases, as it is the immune system's 'nature' to 'defend the body'. Naturally occurring autoantibodies (NAbs) are one such precious gift of nature given to us for our well-being. Previously, we have shown that NAbs contain antibodies against Aβ (Balakrishnan et al., 2010; Dodel et al., 2011; Dodel et al., 2004). Excessive accumulation of misfolded Aβ peptide is one of the main culprits to cause AD. The physiological role of Aβ peptide remains unclear. Aβ, within physiological levels, are essential to perform normal synaptic activities in the brain. Apart from the overproduction of Aβ observed in the familial (early onset) AD patients carrying mutated APP (Chartier-Harlin et al., 1991; Citron et al., 1992; Goate et al., 1991), impaired clearance of normally produced Aβ leads to Aβ accumulation. Decreased levels of Aβ specific autoantibodies in AD patients (Weksler et al., 2002) may therefore lead to Aβ accumulation and are suspected to be one of the causative features of AD (Dodel et al., 2009). On the contrary, some reports suggest that the autoantibodies are increased (Moir et al., 2005; Nath et al., 2003) in AD patients

Apart from the currently available therapies (1.5.1), which offer symptomatic reliefs, there are two disease-modifying approaches to treat AD with respect to amyloid hypothesis. 1. Drugs targeting Aβ production by regulating/inhibiting β or γ-secretase. But recently AD therapeutic research faced a major setback against the γ-secretase inhibiting drug therapy. The γ-secretase inhibitory effect of the drug worsened the cognitive function of patients treated compared to the placebo treated control group (Extance; Lilly, 2010). 2. Vaccination approach to clear Aβ from the brain (1.5.2).

Considering the complexity of Aβ aggregation and role of its conformation in disease, the choice of antibodies becomes crucial. Antibodies recognizing monomeric Aβ peptide sequence does not necessarily become an ideal candidate for passive immunotherapy. Using monoclonal antibodies targeting physiological Aβ or APP products, like sAPPα may lead to adverse effect. As a potential alternative, conformation specific antibodies that could specifically target 'toxic' conformation of Aβ, but not towards other by-products of secretase cleavage may be an effective and safer approach. In this perspective, we proposed NAbs as a possible potential candidate for immunotherapy (Dodel et al., 2002). IVIGs are FDA approved

therapeutic agent to treat immunological disorders like autoimmunity and immunodeficiency. Additionally, there are evidences suggesting that NAbs recognize conformational epitopes on Aβ.

In the first chapter of the discussion, I describe both the region and conformation epitope of Aβ recognized by NAbs-Aβ, and thereby suggesting why it is imperative to use NAbs-Aβ as a therapeutic agent. Characterization of a novel oligomeric species of Aβ raised by artificial double mutation on WT Aβ will be described in the second chapter. Final chapter describes the role of an anti-inflammatory drug on Aβ oligomers and regulation of secretase pathways involved in Alzheimer's disease.

4.1 Presence of Aβ specific autoantibodies in IVIG

Aim of the study is to investigate the Aβ epitope region recognized by NAbs-Aβ. Extraction of Aβ from AD brain showed different variants of post-translationally modified, oxidized and truncated Aβ (Iwatsubo et al., 1996; Mori et al., 1992; Naslund et al., 1994; Piccini et al., 2005). Similarly, human serum pool contains different antibodies which are used to treat immunological diseases also possess Aβ specific antibodies (Dodel et al., 2002). Less is known about the NAbs binding region on Aβ epitope.

First of all, experiments were conducted to compare the levels of Aβ specific antibodies present in different commercial IVIG available in the market. The indirect ELISA results showed all of the IVIGs contain Aβ specific antibodies. Interestingly, some IVIG preparations had differing ratio of antibodies directed towards $A\beta_{1-40}$ (Figure 5) and $A\beta_{1-42}$ (Figure 6). Among all the IVIG brands compared (Table 2), Octagam and Gammunex showed the strongest binding towards $A\beta_{1-40}$ and $A\beta_{1-42}$. Other brands Sandoglobulin, Gammagard and Intratect, but interestingly Kiovig had the lowest concentration of NAbs-Aβ specific to $A\beta_{1-40}$ and showed moderate levels towards $A\beta_{1-42}$. This observation reconfirms the existence of a variety of NAbs in IVIG and in turn serum pool, especially with N and/or C terminal domain specificity. These differences of NAbs among the different IVIG brands suggest that the production strategies and/or composition of storage buffers may influence the levels of Aβ specific NAbs. Although binding affinity towards Aβ is not directly proportional to efficacy of the IVIG, the amount of NAbs-Aβ should be considered before using in AD clinical trials (Balakrishnan et al., 2010).

4.2 Characterization of Aβ epitope recognized by NAbs-Aβ

Throughout the epitope characterization experiments NAbs-Aβ purified from IVIG brand Octagam, were used. Aβ domain specificity of NAbs-Aβ were determined coating different truncated domains of Aβ and tested using indirect ELISA. The comparison showed the C-terminal specific affinity of NAbs-Aβ. Next to the full length $A\beta_{1-40}$, NAbs-Aβ showed better binding to the C-terminal (CT) end compared to the truncated peptides representing N-terminus, mid-terminus (Balakrishnan et al.,

2010). Interestingly, the binding of NAbs-Aβ was less toward the truncated sequence 20-38 (lacks Valine 39 & 40) despite its sequence similarity to 25-40 (Figure 7). This set of data emphasizes the importance of the last two amino acids (Valine 39 and 40) of $A\beta_{1-40}$ for NAbs binding. $A\beta_{1-38}$ is one of the three possible products out of γ-secretase cleavage and relatively less neurotoxic when compared to the other two γ-secretase products. This effect may be because of the hydrophobic Valine at 39 and 40 that triggers oligomerization. In summary, this data provides two possible explanations for NAbs-Aβ binding (i) Valine at 39 and 40 represents the epitope region or (ii) the oligomerization induced by Valine thereby form certain oligomers that facilitates NAbs-Aβ binding. This finding strongly suggests that the NAbs-Aβ recognize the CT end of Aβ. Role of CT end in Aβ oligomerization hints that the NAbs-Aβ's CT specificity may also be due to their binding ability towards oligomers. This observation further supports the previous reports suggesting that the human antibody pool consists of antibodies specific to both N and C-terminal end of Aβ. Human antibody pool or the purified IVIG contains N-terminal specific antibodies that are specific to fibrillar conformation and C-terminal sequence specific antibodies that are conformer specific (Balakrishnan et al., 2010; Britschgi et al., 2009; Dodel et al., 2011; O'Nuallain et al., 2008).

Further characterization using alanine-scanning mutagenesis showed that positions 25 to 27 do not represent the Aβ epitope region. In principle, replacement of original amino acid with alanine abolishes binding of antibodies at that position; this is due to inert property of side chain that cannot act as an epitope. Additionally, exchange of L-Serine to D-Serine at position 26 had no influence on binding of NAbs-Aβ. These data reflect that Glycine at 25, Serine at 26 or Asparagine at position 27 are not part of the Aβ epitope region (Figure 8). Based on these data the amino acid sequence between 28-40 plays a crucial role for the NAbs-Aβ binding.

Lysine at 28^{th} position of Aβ compared to the other Aβ fragments improves the positive charge at physiological pH and was reported to be highly toxic due to the presence of Lys28 at the N-terminus (Fradinger et al., 2008). Additionally, Lys28 is possibly the key amino acid to collapse the cross-beta sheet structure (Numata and Kaplan, 2010). Taking these points into account 3 truncated peptides 26-42, 28-42, and 30-42 were compared for NAbs-Aβ binding. To avoid shortening of the length of

the truncated peptide the C-terminal end (x-42) was preferred instead of x-40 for the NAbs-binding studies. Results showed that NAbs-Aβ recognize $Aβ_{28-42}$ better than $Aβ_{1-40}$, in addition $Aβ_{30-42}$ has decreased binding to NAbs-Aβ . The improved binding may be due to two reasons, either Lys28 is one of the amino acids representing a critical part of the epitope region that NAbs-Aβ recognize or it helps the solubility of the peptide at neutral pH and provides β-sheet conformation due to its positive charge as previously reported (Terzi et al., 1994). Decreased binding of $Aβ_{30-42}$ towards NAbs-Aβ also supports the assumption that $Aβ_{28-42}$ is the crucial epitope region or poor solubility due to lack of the peptide and could therefore technically affect the ELISA to decrease the binding of NAbs-Aβ (Figure 10). Additionally, short truncated version of conformationally fixed peptides that lack Lys 28 were poorly bound by NAbs-Aβ, which will be discussed in detail in the following chapter (4.3). $Aβ_{28-40}$ consists of 4 glycine and one alanine residues which cannot act as epitope due to lack of the functional group. Taken together, these data shortlists about 3 to 6 amino acids in the CT end that could represent Aβ epitope region for NAbs-Aβ binding. Previously, Tycko and Petkova have developed a model using the information they obtained through the studies on $Aβ_{40}$ residues. The model proposes that 9-24 and 30-40 are parallel in-register β-sheets and 23-29 is the loop region stabilizing these β-sheets (Petkova et al., 2002; Petkova et al., 2006) (Figure 47). NAbs-Aβ, which recognize between 28-40 sequences, with respect to the proposed Petkova model, binds to the one of the β-pleated conformation of Aβ. Interestingly, this sequence of amino acid is the membrane bound region of Aβ (Figure 48).

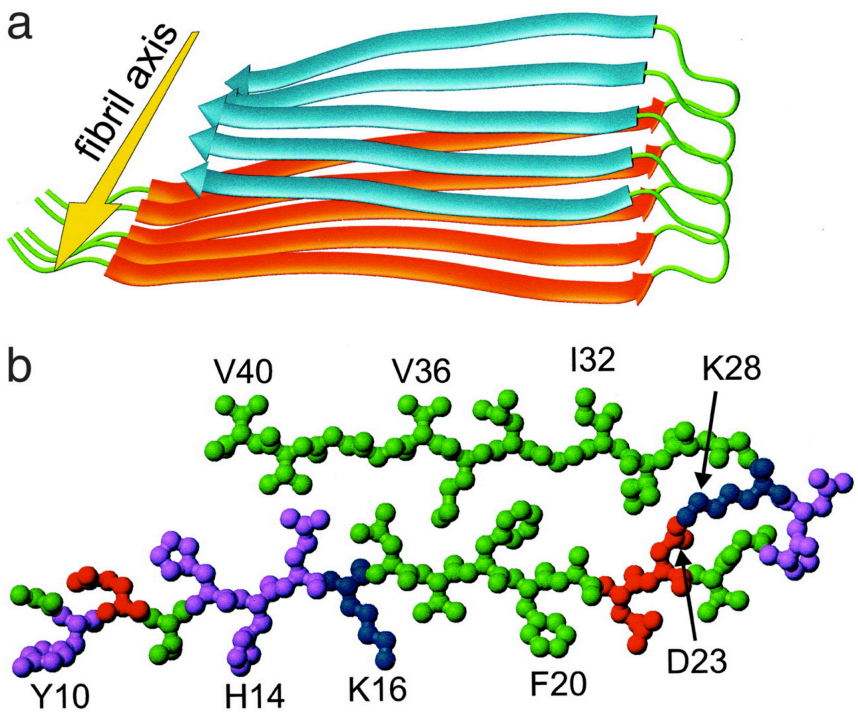

Figure 47 Petkova model (a) Schematic representation of a single molecular layer, or cross-β unit. The yellow arrow indicates the direction of the long axis of the fibril, which coincides with the direction of intermolecular backbone hydrogen bonds. The cross-β unit is a double-layered structure, with in-register parallel β-sheets formed by residues 12–24 (orange ribbons) and 30–40 (blue ribbons). (b) Central Aβ1–40 molecule from the energy-minimized, five-chain system, viewed down the long axis of the fibril. Residues are color-coded according to their side chains as hydrophobic (green), polar (magenta), positive (blue), or negative (red).

Methionine at position 35 could also possibly act as an epitope because it is the only sulphur containing proteinogenic amino acid present in $A\beta_{1\text{-}42}$ sequence. Additionally, Methionine at 35^{th} position is a critical amino acid for oxidative stress and neurotoxic properties of native Aβ peptides (Drake et al., 2003; Yatin et al., 1999). Alanine scan at 35^{th} position did not affect the NAbs-Aβ binding (Figure 11). This indicates that the methionine is not representing the epitope region for NAbs-Aβ.

Apart from methionine and glycine the remaining amino acids in this domain are hydrophobic and may raise the question whether NAbs-Aβ binding towards the Aβ peptide is an unspecific hydrophobic interaction. Interestingly, substitution of hydrophobic Alanine at Gly33 improved the binding signal indicating an increased NAbs-Aβ binding (Figure 11). Further, it has been reported that C-terminal domain of the Aβ peptide, due to its hydrophobic nature, is responsible for the oligomerization

(Jarrett et al., 1993; Numata and Kaplan, 2010). The improved binding of NAbs-Aβ towards mutated peptide, which we propose, is due to the property of NAbs-Aβ recognizing certain 'oligomers'. This could be verified with the data where we could show that specific mutations at 29 and 33rd position forms an oligomeric structure of the Aβ peptide. The oligomerized structure of that Aβ peptide will be discussed in detail in the following chapter (4.5). In addition, the binding of NAbs-Aβ to the C-terminus could inhibit the fibrillation compared to mid-terminal specific antibodies (Figure 44).

The addition of N-terminal cysteine to $Aβ_{1-40}$ improves the binding of NAbs-Aβ. But N-terminal cysteine addition does not influence $Aβ_{1-12}$ binding (Figure 12). This indicates three properties of NAbs-Aβ:

- The addition of cysteine helps in oligomerization (forming cystine disulphide bonds) and thereby improves the binding.
- Despite the presence of cysteine, the poor binding ability of NAbs-Aβ to $Aβ_{1-12}$ show that their interaction was not with cysteine but specific to aggregates of C-terminal Aβ.
- This further clarifies the effect of the oligomerization that improves NAbs-Aβ biding to cys-$Aβ_{1-40}$ better than towards wt $Aβ_{1-40}$ (Figure 12, Figure 18 Figure 21).

Interestingly, the weak binding partner $Aβ_{20-38}$ could interact better with NAbs-Aβ by replacing valine with cysteine at 39th position. $Aβ_{20-38}$ was poorly bound by NAbs-Aβ compared to $Aβ_{25-40}$ despite having similar sequence except 39 and 40. This effect might be due to the lack of hydrophobic valine at positions 39 and 40, and thereby less oligomerization. This shows that 39 or 40th valine residues are not acting as epitope, but helps in oligomerization of the peptide. Alanine scans at three positions on $Aβ_{28-42}$; Ileu31, Leu34 and Val40 shows no decline in the binding signal provided due to NAbs-Aβ interaction (Figure 12).

Taken together, the Aβ epitope recognized by NAbs-Aβ lies between the sequence 28-40. The moderate signal provided by NAbs-Aβ binding towards $Aβ_{30-42}$ shows that lysine at 28 important and helps in stabilizing a structure and exposes the epitope region. Glycine present at positions 29, 33, 37 and 38 of Aβ and alanine at position 30 cannot represent the epitope due to their lack of lateral side chains, which make them inert to interact with antibodies. Alanine scanning shows replacing Ileu31, Leu34, and

Met35 with alanine did not affect the interaction of NAbs towards Aβ (Figure 12). Substitution of cysteine with valine at 39th improves the binding revealing C-terminal valine residues are participating in oligomerization and not one of the epitope representing amino acids. According to the results we obtained, Lys28, Gly29, Ala30, Ile31, Gly33, Leu34, Met35, Gly37, Gly38, Val39 and Val40 are not representing the epitope region. This leaves behind Ile32 or Val36, if the NAbs-Aβ are specific to Aβ, alanine replacement at Ile32 or Val36 or both replaced should abolish the NAbs-Aβ binding.

As expected, there was a remarkable decline in the NAbs-Aβ binding to $A\beta_{28-42}$ with alanine substitutions at Ile32, Met35 and Val40 (Figure 14). Additionally, a scramble sequence (every amino acid except Ile32, Met35 and Val40 were replaced with amino acids of similar physical properties) showed similar NAbs-Aβ binding compared to the WT $A\beta_{1-40}$ (Figure 14). These results clearly demonstrate the role of Ile32, Met35 and Val40 in representing the critical amino acids of the epitope region of NAbs-Aβ.

Furthermore, the unaffected binding signal after exchanging Asp23 and Lys28 shows the loop formation proposed by Petkova *et al* does not play any crucial role in NAbs-Aβ binding (Figure 14). According to the Petkova *et al* model, there lies two parallel β-sheet structures within the mid (12-24) and the C-terminal end (30-40) of Aβ peptide (Figure 47). This supports that the epitope region lies between 30-40 β-sheet structure. Cysteine addition at the C-terminal end moderately decreases the binding of NAbs-Aβ compared to cysteine added to the N terminal or to both ends of the peptide (Figure 14).

Alanine scan exclusively at Ile32 could completely abolish NAbs-Aβ binding (Figure 15). This result nails down the epitope region. Either Ile32 alone or Ile32 in combination with other amino acids completely abolishes NAbs-Aβ binding (Figure 15). We propose, either Ile32 represents the Aβ epitope, or Ile32 helps in the oligomerization of Aβ that effectively unravels the epitope region, for NAbs-Aβ binding. In support of the observation that the oligomerization of Aβ improves NAbs-Aβ binding, a time course oligomerization experiments shows that NAbs-Aβ recognize particularly enriched form of dimers (Figure 18-Figure 21).

4.3 NAbs-Aβ binding towards conformationally fixed peptides

Given the complexity of Aβ aggregation, conformationally fixed ‘‘click’’ peptides (that slows down the aggregation process) were used to identify the right conformation of NAbs-Aβ recognized Aβ epitope. Comparison studies of NAbs-Aβ to different monomeric and dimeric truncated Aβ species demonstrated clearly that NAbs-Aβ preferentially binds to Aβ oligomers. In line with the previous observation, out of 12 conformationally fixed MA peptides, Gly33 replaced with hydrophobic *t-leu*, and $Aβ_{28-40}$ with open flexible (MA 04) and closed (MA 06) dimeric conformation showed improved recognition by NAbs-Aβ (Figure 23-Figure 25). Conformationally fixed peptides re-emphasize the role of Lys28 in NAbs-Aβ recognition. Despite the representation of specific (dimeric) conformations, lack of Lys28 drastically diminishes the NAbs-Aβ binding. The revival of NAbs-Aβ binding upon the inclusion of Lys28 once again supports its role in NAbs-Aβ to Aβ interaction (Figure 27).
Comparison of the promising NAbs-Aβ binding candidates among 24 peptides to wt $Aβ_{1-40}$ and $Aβ_{1-42}$ revealed that NAbs-Aβ preferential binds towards peptides exhibiting dimeric conformation (Figure 28). In addition, parallel dimers, cyclic peptides and truncated 28-40 showed good binding to NAbs-Aβ. This is consistent to the previous observation where Cys-Aβ1-40 that has the tendency to form dimers (Figure 19-Figure 21) showed improved recognition by NAbs-Aβ (Figure 18). These results suggest that the right conformation (parallel, anti-parallel or cyclic) is important along with the presence of Lys28 for the NAbs-Aβ to Aβ interaction (Figure 31).

The same set of peptides probed using C-terminal $Aβ_{1-40}$ specific and mid-terminal specific monoclonal antibodies clearly demonstrates that the antibodies binding was not because of any unspecific molecular clouding effect of the conformationally fixed peptides (Figure 32).

Finally, these results confirmed the binding of NAbs-Aβ has a preferentially improved binding towards anti-parallel flexible dimers. Thus we conclude that the NAbs-Aβ binding is specific to certain conformation and sequence of Aβ. Further, we propose that affinity purification carried out in our laboratories using N-terminal cysteine tagged Aβ could possibly help in isolation of the oligomer specific NAbs-Aβ antibodies from the IVIG preparation. This isolation procedure further helps in

selective isolation of oligomer-specific autoantibodies help from IVIG where both oligomer specific and fibril specific autoantibodies coexist.

4.4 Comparison of NAbs-Aβ vs Aβ specific monoclonal antibodies

Taken together, NAbs-Aβ found in the human sera, contain oligomer specific antibodies according to the results discussed in the previous chapter and consistent with other reports as well as fibril specific antibodies (Britschgi et al., 2009; Dodel et al., 2011; O'Nuallain et al., 2008). It is proposed that in the diseased individuals NAbs-Aβ might be diminished and thereby impairing Aβ clearance, which leads to the disease progression (Dodel et al., 2011). When antibodies are considered for the immunotherapy, it is very important to target exclusively the toxic oligomers and leaving the physiological Aβ undisturbed. The results in this current work clearly demonstrates that affinity purified antibodies recognize low-molecular weight oligomers of Aβ and does not interact with neither fibril conformation nor the monomers that are required for physiological neuronal functions. When compared with other antibodies, NAbs-Aβ recognize the dimeric Aβ species (Figure 18), (Figure 43) and β-sheet enriched trimer and has no interaction with $Aβ_{1-40}$ and binds to $Aβ_{1-42}$ monomers or to the break down products (Figure 15). On the other hand, oligomer specific A11 antibodies do recognize low-molecular weight forms of Aβ, here again, these may be break down products of the oligomers which were disturbed while boiling the samples (Figure 43). Interestingly $Aβ_{1-40;G29,33Ile}$ mutants could display the trimeric structure unaffected or reformed despite the boiling step after Immunoprecipitation, this implies either the trimeric structure is stable or the mutation tend to form trimers in solution form without any incubation steps. The Aβ toxicity is supposed to be due to the presence of SDS-stable oligomers. On the contrary, $Aβ_{1-40;G29,33Ile}$ mutants, despite being SDS-stable are non-toxic in our experiments. 3D6 antibodies recognize all the species except N-terminal cysteine added $Aβ_{1-40}$. N-terminal specific monoclonal antibody 3D6 might not be the best option for immunotherapy considering its strong binding ability towards the physiological importance of monomeric Aβ and could also bind to the neuroprotective sAPP-α. Comparing m266, NAbs-Aβ could stop the fibrillation at its initial stages (Figure 44).

We hypothesize that NAbs-Aβ were either produced to target oligomeric forms of Aβ or the p3 region (24-42) released subsequent to α-secretase cleavage. We also propose that the role of NAbs-Aβ is to clear the accumulation of p3, due to aging when the NAbs-Aβ are decreased and there might be a switch from α-secretase cleavage (non-amyloidogenic) to β-secretase cleavage pathway (amyloidogenic). Schematic diagram explains comparing the NAbs-Aβ, 3D6 and m266 binding region (Figure 48).

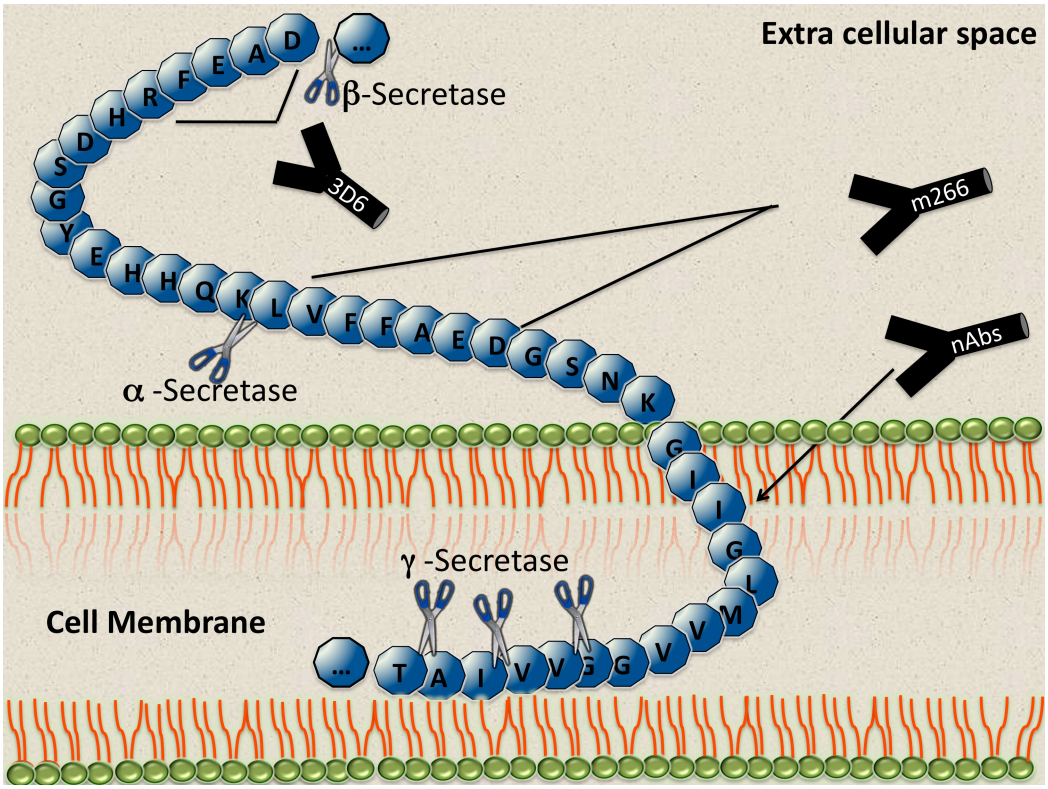

Figure 48 Schematic diagram: of secretase activities and antibody binding domain on Aβ peptides

4.5 Characterization of oligomers of $A\beta_{1\text{-}40;G29,33Ile}$

Previously, it was shown that NAbs-Aβ recognized a double mutant form of Aβ peptide (4.2)(Figure 34), and preferentially improved binding towards oligomers of Aβ (4.3). Interestingly, the substitution of isoleucine at Gly29 and Gly33 of $A\beta_{1\text{-}40}$ instantly triggered the formation of stable low-molecular weight oligomers (Figure 33). In $A\beta_{1\text{-}42}$ the double substitution of isoleucine at Gly29 and Gly33 forms higher aggregates, whereas in $A\beta_{1\text{-}40}$ the aggregation stops after tetramer formation. This effect shows the crucial role of those two CTE amino acids in determining the aggregation pattern. The importance of GXXXG was reported by Muthaulp *et al* where they claim that the GXXXG domain responsible for APP dimerization (Munter

et al., 2007). Further, they could show that replacing Gly29 or Gly33 with alanine or isoleucine prevents APP dimerization. On the contrary, our observation proposes another hypothesis that the mutations at GXXXG domain break the helical structure provided by the glycine residues by substituting with hydrophobic amino acid isoleucine.

Toxicity assays confirm that the wild type and the mutant peptides are non-toxic in the freshly prepared-monomerized conditions both on neuronal and microglial cell lines (Figure 35-Figure 38). Several reports claim that the anti-inflammatory agents help to fight against AD (McGeer et al., 1990). In this toxicity experiment, the treatment of an anti-inflammatory drug CNI-1493 helped the cell survival against the Aβ induced toxicity.

In line with the previous studies from our laboratory (Bach et al.) and Kayed's report of oligomerization of wt $A\beta_{1-40}$ and $A\beta_{1-42}$ using their protocol (Kayed et al., 2003), we induced a toxic effect on both used cell lines and treatment of CNI-1493 could improve the cell survival (Figure 39), (Figure 41). Surprisingly, the double mutants $A\beta_{1-40;G29,33Ile}$ and $A\beta_{1-42;G29,33Ile}$ showed no observable toxic effect on the cells in the same oligomerization conditions (Figure 40) (Figure 42). These data indicates that the mutants $A\beta_{1-40;G29,33Ile}$ and $A\beta_{1-42;G29,33Ile}$ follow a different oligomerization pattern and does not induce cell toxicity in these oligomerization conditions. One possible explanation for this lack of toxicity is that the aggregation rate and growth of oligomers are more crucial to exert Aβ toxicity than a stable oligomeric form of Aβ (Jan et al.). These results also demonstrate that the CNI-1493 specifically binds to toxic conformation of the peptide. The effect of CNI-1493 on Aβ oligomers will be dealt in details in the following chapter (4.6).

4.6 Effect of an anti-inflammatory drug CNI-1493 on Aβ oligomers

Anti-inflammatory drug CNI-1493 could reduce the plaque burden and improve cognition in APP over-expressing transgenic mice model (Bacher et al., 2008). CNI-1493 that is already in clinical phase II research for Corn's disease has MAP-kinase inhibitory action. Previous studies in our laboratory demonstrated that CNI-1493 decreases the plaque deposition and improves cognition in transgenic animals mimicking Alzheimer's disease.

Previously the protective effect of CNI-1493 was shown to rescue cells from Aβ induced cytotoxicity (Figure 39). In this study, CNI-1493 showed a destabilizing effect on the toxic oligomeric conformation of Aβ peptide. Western blotting results supports this proposal as the Aβ oligomers electrophoresed in the presence of CNI-1493 could break the SDS-stable oligomers and increase the monomers and low-molecular weight Aβ (Figure 45). A11 antibodies were raised against conformational epitope of Aβ (Kayed et al., 2003). Dot blotting with oligomer specific A11 antibodies showed that the toxic conformation of the peptide is lost in a concentration dependent manner (Figure 46). These data suggest that CNI-1493 might also be effective, not only with its anti-inflammatory nature but also, against Aβ oligomers and its toxic conformation.

4.7 FUTURE PERSPECTIVES

In this thesis, the characterization of the crucial Aβ epitope of naturally occurring autoantibodies, characterization of a novel Aβ oligomer, and effects of a non-steroidal anti-inflammatory drug on crucial players of AD were investigated. It was shown that naturally occurring autoantibodies contain antibodies against a specific conformational Aβ epitope. Despite the enormous amount of efforts taken by scientific communities both in academia and bio-pharmaceutical industries, there are very few drug candidates entering clinical phases. Recently, there are some dropouts from clinical phase III trials due to adverse effects. Alarmingly, at this point of time there is an urgent need for drugs that are efficient and safe to use. In this context, the naturally occurring conformation specific autoantibodies could be of great help to develop a future antibody treatment for AD. Although, the IVIG immunotherapy for AD has successfully entered the confirmatory phase of the clinical phase III, considering the number of AD patients and cost involved in the IVIG preparations it is very important to look for alternatives approaches. Considering these aspects, deriving monoclonal antibodies using reverse translational approaches from the conformational autoantibodies against Aβ might be a promising approach that could be cost effective and very efficient. Among the compared Aβ species, Aβ double mutant trimers show highest binding towards NAbs-Aβ and thus might become a promising candidate for active vaccination for AD that might raise conformation specific antibodies in healthy individuals to protect them from AD risk. In addition, the $A\beta_{1\text{-}40;G29,33Ile}$ trimers could also be used for identifying the B-cells that contain the natural antibodies against oligomeric Aβ and thereby useful to produce monoclonal antibodies for immunotherapy approaches. The high affinity of NAbs-Aβ towards $A\beta_{1\text{-}40;G29,33Ile}$ trimers could also be exploited to develop an assay to measure oligomer specific autoantibodies present in the human sera. This assay will therefore be useful to speculate the subjects having low oligomer specific antibodies having more chance of developing AD and additionally, this assay could also be used to monitor the improvement among subjects receiving Aβ active immunotherapy for AD clinical trials. Neuroinflammation has been reported to be involved in the progression of AD. CNI-1493, a non-steroidal anti-inflammatory was shown to be effectively against toxic Aβ oligomers that are closely associated with AD. Thus, CNI-1493, which is currently in the clinical trial phase II for Corhn's disease could be effective against AD as well.

5 SUMMARY

SUMMARY

The broad objective of this study is to unravel the potential effect of naturally occurring autoantibodies (NAbs) and CNI-1493 (immunomodulatory compound) on Amyloid beta peptide (Aβ) oligomers. Extracellular accumulation of Aβ peptide in the brain is suspected to cause and trigger the progression of Alzheimer's disease (AD). The exact physiological role of Aβ remains unclear. The oligomeric forms Aβ impair synaptic activity. Hence, immunotherapeutic approach targeting Aβ is one of the promising strategies to treat AD. One of the challenges in the immunotherapy approach is to specifically target the toxic misfolded forms of Aβ without disturbing the physiological levels of Aβ monomers. Naturally occurring autoantibodies against Aβ are reduced in AD patients.

In the first chapter, I have characterized the Aβ epitope region of Aβ specific-naturally occurring autoantibodies (NAbs-Aβ) using binding studies. I compared the binding ability of NAbs-Aβ towards a variety of mutated and truncated Aβ peptides. NAbs-Aβ had greater binding ability towards the C-terminal sequence of Aβ, which is membrane bound. The C-terminal end of Aβ is crucial for the formation of Aβ oligomerization. Hence, C-terminal specificity of NAbs-Aβ could help in the minimizing of Aβ oligomerization. Additionally, this specificity also rules out the possible interactions of NAbs-Aβ with membrane bound amyloid precursor protein (APP), Aβ monomers and fibrils. Further studies on Aβ epitope characterization of NAbs-Aβ were performed using site-directed mutagenesis. The results indicated that the lysine at 28th and isoleucine at 32nd position of Aβ are crucial for the binding of NAbs-Aβ. Deletion or substitution of any of these two amino acids at their respective positions lead to loss of binding. Additional binding studies using conformationally fixed 'click' Aβ peptides indicated that the NAbs-Aβ are specific for low-molecular weight anti-parallel dimers. Interestingly, NAbs-Aβ showed excellent binding towards Aβ with two substitution mutations at C-terminal ($Aβ_{1-40;G29,33Ile}$) end. In the second chapter, characterization of $Aβ_{1-40;G29,33Ile}$ showed that the peptide undergo a different oligomerization process to form SDS-stable trimers. Cytotoxicity studies showed that Aβ wild type forms toxic oligomers at certain incubation conditions where as $Aβ_{1-40;G29,33Ile}$ remains non-toxic under the same conditions. In-line with the results,

according to my current understanding, $A\beta_{1\text{-}40;G29,33Ile}$ is the first oligomeric Aβ species that is stable and has high affinity to NAbs-Aβ.

Non-steroidal anti-inflammatory drugs are reported to provide beneficial effects in AD. In the third chapter, I have investigated the role of CNI-1493, which is an anti-inflammatory drug. *In vitro* studies show that CNI-1493 binds to Aβ oligomers and destabilizes the complex to monomers.

In this study, I have characterized in detail, the effect of two immune system related AD drug candidates, NAbs-Aβ and CNI-1493, on Aβ oligomers. These findings help our understanding about the NAbs-Aβ and support their potential role as a safe and efficient AD immunotherapy candidates. The immunomodulatory effect combined with its role on Aβ oligomers make CNI-1493 an interesting drug candidate for AD therapeutic studies. In addition, the novel Aβ oligomer could be developed as a potential vaccine and diagnostic tool for NAbs-Aβ after further characterization.

6 ZUSAMMENFASSUNG

Das umfassende Ziel dieser Arbeit ist es die Effekte von natürlich vorkommenden Autoantikörpern (Nabs) und CNI-1493 (immunmodulatorische Substanz) auf Amyloid-beta (Aβ) Oligomere zu beschreiben. Die extrazelluläre Anreicherung von Aβ im Gehirn steht im Verdacht Ursache und Auslöser der Alzheimer'schen Demenz (AD) zu sein. Die genaue physiologische Rolle von Aβ ist jedoch unklar. Jüngere Ergebnisse zeigen dass monomeres Aβ an der normalern synaptischen Aktivität beteiligt sein könnte. Im Gegensatz dazu behindert oligomeres Aβ die synaptische Aktivität. Daher ist ein immuntherapeutischer Ansatz, der Aβ zum Ziel hat, eine vielversprechende Behandlungsstrategie für AD. Eine der Herausforderungen eines immuntherapeutischen Ansatzes ist es spezifisch die toxischen, fehl-gefalteten Formen von Aβ zu bekämpfen ohne die physiologisch relevanten Aβ Monomere zu stören. Die Menge an natürlich vorkommenden Autoantikörpern gegen Aβ ist in AD Patienten erniedrigt.

Im ersten Kapitel habe ich die Aß-Epitop-Region von natürlich vorkommenden Aβ-Autonatikörpern (Nabs-Aβ) mit Bindungsstudien ermittelt. Ich verglich die Bindung von Nabs-Aβ gegenüber einer Reihe von mutierten und verkürzten Aβ-Peptiden. Die Nabs–Aβ zeigten eine stärkere Bindung zum C-terminalen Ende von Aβ, das membrangebunden vorliegt. Das C-terminale Ende von Aβ ist entscheidend für die Oligomerisation. Deshalb könnten C-terminal-spezifische Nabs-Aβ helfen die Oligomerisierung zu minimieren. Zusätzlich könnte diese Spezifität eine Interaktion von nabs-Aβ mit membrangebundenen Amyloid-Vorläufer-Protein (APP), Aβ-Monomeren, und Aβ-Fibrillen ausschließen. Weitere Studien zur Epitop-Charakterisierung wurden mit ortsgerichteter Mutagenese durchgeführt. Die Ergebnisse zeigten dass Lysin an Position 28 und Isoleuzin an Position 32 von Aβ entscheidend für die Bindung von Nabs-Aß sind. Das Fehlen oder Ersetzen einer dieser beiden Aminosäuren führte zum Bindungsverlust. Zusätzliche Bindunsgstudien mit konformatorisch fixierten sogenannte Aβ-click-Peptiden zeigten dass nabs-Aβ spezifisch für niedermolekulare anti-parallele Aβ Dimere sind. Interessanterweise zeigten Nabs-Aβ eine exzellente Bindung mit Aβ mit 2 Muationen am C-terminalen Ende ($A\beta_{1\text{-}40;\ G29,33\ Ile}$). Die Charaktersisierung von $A\beta_{1\text{-}40;\ G29,33\ Ile}$ zeigte, dass das Molekül einen unterschiedlichen Oligomerisierungsprozess durchläuft und SDS-stabile Trimere bildet. Zytotoxizitäts-Studien zeigten dass Aβ-Wildtyp unter

bestimmten Inkubationsbedingungen toxische Oligomere bildet, während $A\beta_{1\text{-}40;\ G29,33\ Ile}$ unter diesen Bedingungen nicht toxisch ist. Entsprechend meines derzeitigen Kenntnisstands ist $A\beta_{1\text{-}40;\ G29,33\ Ile}$ die erste Aβ-Oligomer-Spezies die stabil ist und eine hohe Affinität zu Nabs-Aβ besitzt.
Nicht-steroidale ani-inflammatorische Heilmittel haben Berichten zufolge einen positiven Behandlungs-Effekt bei AD. Ich untersuchte in dieser Arbeit die Rolle von CNI-1493, einer anti-inflammatorischen Verbindung. Die in vitro Experimente zeigten dass CNI-1493 Aß-Oligomere bindet und den Komplex in Richtung Monomere verschiebt.

In dieser Arbeit habe ich im Detail die Effekte von zwei AD-Heilmittel-Kandidaten untersucht, die mit dem Immunsystem assoziiert sind; Nabs-Aβ und CNI-1493. Die Ergebnisse helfen unserem Verständnis über Nabs-Aβ und unterstützen ihre mögliche Rolle als sicheren und effektiven AD-Immuntherapie-Kandidaten.
Der immunmodulatorische Wirkung von CNI-1493, sowie seine Effekte auf Aβ-Oligomerbildung machen dieses Molekül zu einem interessanten Arzeinmittel Kandidaten für AD Therapie-Studien. Zusätzlich könnte die neue Aβ-Mutante, nach weiterer Charakterisierung, als mögliche Vakzine oder diagnostisches Verfahren entwickelt werden.

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8 APPENDIX

8.1 Abbreviations

Aa	Amino acids
Aβ	Amyloid beta
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
APLP	APP like protein
APP	Amyloid precursor protein
BACE	β-site APP-cleaving enzyme
BSA	Bovine serum albumin
APP_{CTF}	APP C-terminal fragment
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DTT	1,4 Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbant assay
FAD	Familial Alzheimer's Disease
Fig.	Figure
IL	Interleukin
IP	Immunoprecipitation
IVIG	Intravenous immunoglobulin
kDa	Kilo Dalton
LTP	Long term potentiation
MS	Mass spectromertry
M.Wt	Molecular weight
NAbs	Naturally occurring autoantibodies
NAbs-Aβ	Affinity purified Aβ specific-NAbs
NICD	Notch intracellular domain
NTF	N-terminal fragment
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

rpm	Revolutions per minute
sAPPα	Soluble APP alpha
sAPPβ	Soluble APP beta
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TRIS	Tris-(hydroxymethyl)-aminomethane
TNF-α	Tumor necrosis factor-α
wt	Wild type

Single letter codes were used for amino acid designation.

8.2 Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer in Salem, Madurai und in Marburg waren die Damen und Herren Professoren, Doktoren und Dozenten

Anandbabu
Bacher
Buddhan
Dhamodar
Dodel
Emmanuel
Geyer
Jayaraman
Jaabir
Kalaiselvi
Kandula
Latha
Mohana
Müller
Oertel
Priya
Rajamanickam
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Venkataraman

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8.4 Curriculum Vitae

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Peer-reviewed publications

Dodel, R., **Balakrishnan, K**., Keyvani, K., Deuster, O., Neff, F., Andrei-Selmer, L.C., Röskam, S., Stüer, C., Al-Abed, Y., Noelker, C., Balzer-Geldsetzer, M., Oertel, W., Du, Y., Bacher, M.
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Epitope mapping of naturally occurring antibodies to amyloid-β peptide of Alzheimer's disease.

Balakrishnan, K*., Büttner, C*., Stanek, A.M., Klein, C., Ebert, U., Hillen, H., van der Kam, E., Barghorn, S.
Appearance of Aβ oligomers in canine brains - a non-transgenic model to study Alzheimer's disease.
* Equal contribution.

Patents

A method, particularly an enzyme-linked immunosorbent assay (ELISA), for the *in vitro* detection of Aβ autoantibodies, microtiter plate and test kit.
Germany Patent Application PCT/DE2010/000817 Filed July 14, 2010. Inventors: **Karthikeyan Balakrishnan**, Michael Bacher, and Richard Dodel

Diagnosis, prophylaxis and therapy for Alzheimer's disease and other neurodementing disorders.
United States Patent Application 61362414 Filed July 8, 2010. Europe Patent Application 10168867.9 Filed July 8, 2010. Inventors: **Karthikeyan Balakrishnan**, Michael Bacher, and Richard Dodel

Synthetic peptides ('Mini-amyloids')
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Scientific posters

Balakrishnan, K., Mengel, D., Neff, F., Al-Abed, Y., Bacher, M., Dodel, R.C. "The anti-inflammatory drug CNI-1493 decreases the toxic amyloid oligomers and induces neuro-protective effect in APP transgenic mice." *International Conference on Alzheimer's Disease 2009, Vienna*

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Oral presentation in *National Symposium on Bio-medicine and Molecular Remodeling October, ANJA college, India 2001* entitled "Gene replacement therapy in the Central Nervous System: Viral vector mediated therapy for global neurodegenerative disease"

8.5 Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotion eingereichte Arbeit mit dem Titel „Alzheimer's disease: Amyloid oligomers, therapeutic agents, their targets, and mode of action" selber und ohne fremde Hilfe verfasst, nicht andere als die angegebenen Quellen oder Hilfsmittel benutzt, alle vollständig oder sinngemäß übernommenen Zitate als solche gekennzeichnet sowie die Dissertation in der vorliegenden oder einer ähnlichen Form noch bei keener anderen in- oder ausländischen Hochschule anlässlich eines Promotionsgesuchs oder zu anderen Prüfungszwecken eingereicht habe. Ich habe bischer an keiner Hochschule ein Gesuch um Zulassung zur Promotion eingereicht.

Marburg Karthikeyan Balakrishnan